MOLECULAR AND CELLULAR BIOLOGY, June 2006, p. 4612–4627 Vol. 26, No. 12
0270-7306/06/$08.00+0 doi:10.1128/MCB.02061-05

The Rho GTPase Effector ROCK Regulates Cyclin A, Cyclin D1, and $p27^{Kip1}$ Levels by Distinct Mechanisms

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Received 24 October 2005/Returned for modification 28 November 2005/Accepted 6 April 2006

The members of the Rho GTPase family are well known for their regulation of actin cytoskeletal structures. In addition, they influence progression through the cell cycle. The RhoA and RhoC proteins regulate numerous effector proteins, with a central and vital signaling role mediated by the ROCK I and ROCK II serine/threonine kinases. The requirement for ROCK function in the proliferation of numerous cell types has been revealed by studies utilizing ROCK-selective inhibitors such as Y-27632. However, the mechanisms by which ROCK signaling promotes cell cycle progression have not been thoroughly characterized. Using a conditionally activated ROCK-estrogen receptor fusion protein, we found that ROCK activation is sufficient to stimulate G_1/S cell cycle progression in NIH 3T3 mouse fibroblasts. Further analysis revealed that ROCK acts via independent pathways to alter the levels of cell cycle regulatory proteins: cyclin D1 and $p21^{Cip1}$ elevation via Ras and the mitogen-activated protein kinase pathway, increased cyclin A via LIM kinase 2, and reduction of $p27^{Kip1}$ protein levels. Therefore, the influence of ROCK on cell cycle regulatory proteins occurs by multiple independent mechanisms.

Rho family GTPases contribute to the regulation of many different biological processes, including cell cycle progression, with RhoA, Rac1, and Cdc42 being the best-studied members (12). When bound to GTP, these proteins recruit effector proteins that relay signals downstream and mediate biological responses. RhoA and RhoC activate numerous effector proteins, with the serine/threonine kinases ROCK I (also called ROKβ) and ROCK II (ROKα or Rho kinase) (55) being the most extensively characterized. When activated by association with Rho-GTP, ROCK phosphorylates a number of substrates, such as LIM kinase 1 (LIMK1) and LIMK2 and regulatory myosin light chains (MLC2). Through these phosphorylation events, ROCK promotes the stabilization of filamentous (F) actin and increased myosin ATPase activity, leading to the formation of contractile actin-myosin bundles (often called stress fibers) and integrin-containing focal adhesions (41, 75).

Although Rho GTPases have been implicated in human cancer (21, 60), Rho mutations are not a mode of activation. However, RhoA and RhoC protein levels are significantly elevated in a variety of tumors (21, 32, 38, 60). In addition to elevated RhoA and RhoC, increased levels of ROCK I and/or ROCK II have been found in esophageal squamous cell carcinoma (87) and in testicular germ cell (32), pancreatic (36), and bladder (33) tumors. Although it has been proposed that increased expression of the Rho and ROCK proteins contributes to the metastatic behavior of some cancers (e.g., see reference 18), elevated signaling through the ROCK pathway may also promote tumor cell proliferation. Transformation of NIH 3T3 cells by oncogenic Ras was found to be blocked by the ROCK-selective inhibitor Y-27632, while activated ROCK cooperated with the Ras effector Raf-1 to promote transformation (59). In addition, proliferation of C6 glioma cells (10), HSQ-89 oral squamous carcinoma cells (48), IMGE-5 gastric epithelial cells (25), umbilical vein endothelial cells (66), vascular smooth muscle cells (34, 44, 63, 65), prostatic smooth muscle cells (53), atrial myofibroblast cells (52), cardiac myocytes (86), glial cells (68), spleen-derived primary and Jurkat T cells (45, 74), $CD34^+$ hematopoietic stem cells (77), corneal stromal cells (24), chondrocytes (80), and hepatic stellate cells (30) was inhibited by the ROCK inhibitor Y-27632. Although it has been reported that ROCK activity is required for the formation of actin stress fibers that contribute to the sustained activation of Ras and the ERK mitogen-activated protein kinase (MAPK) following ligand stimulation (57, 71, 83), the possibility remains that elevated ROCK signaling might promote cell cycle progression via additional mechanisms.

The eukaryotic cell cycle is composed of the first gap phase (G_1), the DNA-synthetic phase (S), the second gap phase (G_2), and mitosis (M). Progression through G_1 to S phase is controlled by the cyclin-dependent kinases (CDKs) in association with cyclin regulatory subunits (67). Type D cyclins (D1, D2, and D3) form complexes with CDK4 or CDK6, while cyclin E and cyclin A work in combination with CDK2. The cyclin D-CDK and cyclin E-CDK2 complexes are generally thought to act to promote passage through G_1, while cyclin A-CDK2 contributes to passage through the G_1/S transition and progression through S phase. The activities of cyclin-CDK complexes are modulated by two types of CDK inhibitors (CDKIs) that have differing mechanisms of action. The INK4 CDKI proteins ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$) directly interact with CDK and inhibit its activity. In contrast, the Cip/Kip family CDKIs ($p21^{Waf1/Cip1}$ [p21], $p27^{Kip1}$ [p27], and $p57^{Kip2}$) bind to cyclin-CDK complexes. At high levels, p21 and p27 inhibit cyclin E-CDK2 activity, leading to cell cycle arrest. At lower stoichiometry, however, p21 and p27 promote the assembly, stability, and nuclear retention of cyclin D-CDK4 and cyclin D-CDK6 complexes, which are inefficiently inhib-

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ited by associated Cip/Kip proteins. Cyclin D-CDK4 and cyclin D-CDK6 complexes also relieve cyclin E-CDK2 complexes from Cip/Kip-mediated inhibition by acting as a sink for p21 and p27. Therefore, the relative levels of cyclin, CDK, and CDKI proteins are critical factors that determine whether a cell will progress through G_1 toward S phase.

Initial indications that Rho contributed to cell cycle regulation were the observations that Rho inactivation with the *Clostridium botulinum* C3 exoenzyme blocked serum-stimulated DNA synthesis and that microinjection of active RhoA was sufficient to induce G_1/S-phase progression in Swiss 3T3 fibroblasts (49, 85). In addition, expression of Rho from *Aplysia californica* was found to oncogenically transform NIH 3T3 cells (51). However, the means by which elevated ROCK signaling might promote cell cycle progression, and possibly transformation, have not been thoroughly characterized. In this report, we show that stimulation of a conditionally activated ROCK-estrogen receptor fusion protein (ROCK-ER) is sufficient to stimulate G_1/S cell cycle progression in NIH 3T3 cells. Further analysis revealed that ROCK acts via independent pathways to elevate cyclin D1 and p21 by Ras and MAPK activation, to elevate cyclin A expression via LIMK and to reduce p27 protein levels. Therefore, the influence of ROCK on cell cycle regulatory proteins occurs by multiple mechanisms.

MATERIALS AND METHODS

Antibodies. Antibodies to MEK1/2 (9122), phospho-MEK1/2 (Ser217/221) (9121), phospho-MLC (Ser19) (3671), phospho-MLC (Thr18/Ser19) (3674), LIMK1 (3842), and phospho-LIMK1 (Thr508)/LIMK2 (Thr505) (3841) were from Cell Signaling Technologies (Beverly, MA). Antibodies against myosin light chain (M4401) and phospho-ERK (M8159) were from Sigma (St. Louis, MO). Antibodies against ERα (sc-543), cdk2 (sc-163), cdk4 (sc-260), cdk6 (sc-177), cyclin A (sc-596), cyclin A1 (sc-15383), cyclin D1 (sc-450), cyclin D3 (sc-6283), cyclin E (sc-481), lamin A/C (sc-6215), LIMK2 (sc-5577), p15 (sc-613), p18 (sc-865), p21 (sc-397G), and p107 (sc-318) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against cdk2 (06-505), cyclin E (07-687), focal adhesion kinase (FAK) (06-543), and Ras (05-516) were from Upstate (Lake Placid, NY). Antibodies against paxillin (610051) and p27 (610241) were from BD Biosciences (San Diego, CA). Additional antibodies used were against ROCK II (ROKα, ab24843; Abcam, Cambridge, United Kingdom), cyclin D2 (RDI-CYCLD2abm-43; Research Diagnostics, Flanders, NJ), and phospho-FAK (Tyr397) (44-624; Biosource, Camarillo, CA). Anti-ERK2 antibody (Ab122) was provided by C. J. Marshall (Institute of Cancer Research, London, United Kingdom). Goat anti-mouse, goat anti-rabbit, and rabbit anti-goat horseradish peroxidase-conjugated antibodies were from Pierce (Rockford, IL).

Generation of KD-ER and ROCK-ER cell lines. Retroviral constructs for conditionally regulated ROCK II-ER (ROCK-ER) and the kinase-dead version (KD-ER) were constructed as described previously (17, 18). pBABE puro ROCK-ER and KD-ER plasmids were transfected into BOSC 23 ecotropic retroviral packaging cells with Effectene (QIAGEN) according to the manufacturer's instructions. After 36 h, supernatant was collected and centrifuged at 1,600 rpm for 15 min, and aliquots were stored at −80°C. Exponentially growing NIH 3T3 cells were infected with undiluted retroviral supernatant mixed with 4 μg/ml Polybrene (Sigma) and selected with 2.5 μg/ml puromycin (Sigma) to establish transduced pools.

Cell culture and treatments. Parental NIH 3T3 cell lines or those expressing either kinase-dead KD-ER and ROCK-ER were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% donor calf serum (DCS; Gibco, Paisley, United Kingdom) at 37°C and 10% CO_2. For most experiments, 0.5×10^6 cells were plated onto 100-mm cell culture dishes containing DMEM plus 10% DCS. After 24 h, cells were cultured in serum-free DMEM (serum starved) and treated with or without 4-hydroxytamoxifen (4-HT; 1 μM; Sigma) for 16 h in the presence or absence of pharmacological inhibitors. Inhibitors used were U0126 (Promega) dissolved in dimethyl sulfoxide and used at 10 μM and Y-27632 (Calbiochem) dissolved in water and used at 10 μM. Biocoat fibronectin-coated and poly-D-lysine (PDL)-coated plates were purchased from Becton Dickinson.

For suspension studies, nearly confluent NIH 3T3 ROCK-ER fibroblasts grown in DMEM plus 10% DCS were trypsinized and then placed into suspension in serum-free medium. Cells (1.5×10^6) in serum-free medium were plated onto 100-mm cell culture dishes coated with poly-hydroxyethyl-methacrylate (poly-HEMA; Sigma) and treated with or without 4-HT, in the presence or absence of either Y-27632 (10 μM) or UO126 (10 μM), for 15 h. For studies with actin-disrupting compounds, nearly confluent NIH 3T3 ROCK-ER fibroblasts grown in DMEM plus 10% DCS were trypsinized and 0.5×10^6 cells were plated onto 100-mm cell culture dishes. After 24 h, cells were serum starved and treated with or without 4-HT (1 μM), in the presence or absence of cytochalasin D (CCD; 1 μM; Sigma), latrunculin B (LTB; 0.5 μM; Calbiochem), swinholide A (SWA; 0.05 μM; Sigma), or jasplakinolide (Jasp; 0.5 μM; Molecular Probes), for 16 h.

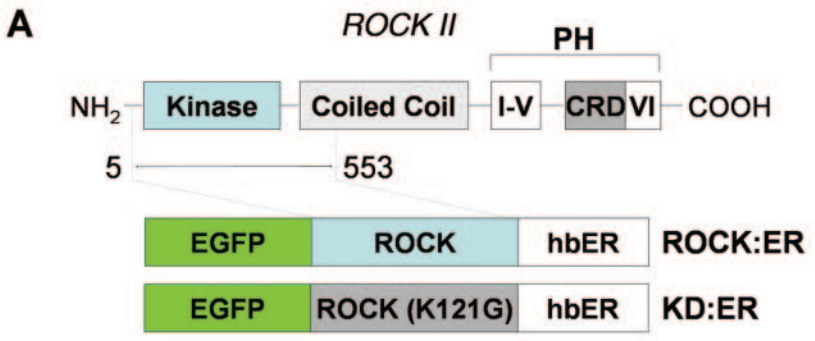
Cell extraction and immunoblotting. Following treatment as described above, cells were washed with phosphate-buffered saline (PBS) and then lysed in buffer containing 10 mM Tris (pH 7.5), 5 mM EDTA, 1% (vol/vol) NP-40, 0.5% (wt/vol) sodium deoxycholate, 40 mM sodium pyrophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.025% (vol/vol) sodium dodecyl sulfate (SDS), 150 mM NaCl, and protease inhibitors. Lysates were clarified by centrifugation at $13{,}000 \times g$ for 15 min. Sixty micrograms of each whole-cell lysate was electrophoresed on SDS-polyacrylamide gel before electrotransfer to nitrocellulose membrane. Blots were probed with antibodies (see above) and appropriate horseradish peroxidase-conjugated secondary antibodies (Pierce), followed by visualization by ECL (Amersham Pharmacia) or SuperSignal West Femto (Pierce) according to the manufacturer's instructions and exposure to Kodak BioMax autoradiographic film. Alternatively, for determining the levels of MLC and phospho-MLC, cells were lysed directly in 3× Laemmli sample buffer. Samples were sonicated and boiled for 5 min, and the supernatant was clarified by centrifugation at $16{,}000 \times g$ for 5 min. An appropriate volume of each sample was electrophoresed and immunoblotted as described above.

Measurement of Ras activation. Following treatment as described above, cells were washed with PBS and then lysed by scraping in MLB buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 1% [vol/vol] IGEPAL [CA-630], 1 mM EDTA [pH 8.0], 10 mM $MgCl_2$, 10% [vol/vol] glycerol, 1 mM Na_3VO_4, 25 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cleared lysates were incubated for 45 min at 4°C with glutathione-agarose beads coupled to glutathione *S*-transferase–Raf-1 RBD (Upstate). The beads were washed three times with MLB buffer, and bound proteins were solubilized by boiling with 60 μl of 3× Laemmli buffer and separated by SDS-polyacrylamide gel electrophoresis. Ras-GTP and total Ras were detected by Western blotting with an antibody against Ras.

Immunofluorescence. Cells were fixed for 15 min in 4% (wt/vol) paraformaldehyde (PFA)–PBS and then permeabilized for 15 min in 0.5% Triton X-100–PBS. After fixation and permeabilization, cells were washed three times in PBS and then blocked with 2% (wt/vol) BSA–PBS for 1 h. Cells were incubated with primary antibodies (1:1,000 dilution) for 60 min, followed by three washes with 2% BSA–PBS and a 60-min incubation with the corresponding fluorochrome-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a 1:200 dilution. Filamentous actin structures were stained with a 1:250 dilution of Texas Red-conjugated phalloidin (T7471; Molecular Probes, Eugene, OR). For visualization of focal adhesions, cells were fixed and permeabilized in one step with 4% PFA–0.2% Triton X-100–PBS. Primary antibodies were as follows: rabbit anti-MLC (Ser19) (3671; Cell Signaling Technologies), mouse anti-cyclin D1 (sc-450; Santa Cruz), goat anti-lamin B1 (sc-6217; Santa Cruz), and mouse anti-paxillin (610051; BD Biosciences). Coverslips were mounted in Mowiol and visualized with a Bio-Rad MRC1024 confocal microscope.

BrdU analysis by fluorescence-activated cell sorter (FACS). For cell cycle analysis, serum-starved NIH 3T3 ROCK-ER cells were left untreated or treated with 4-HT (1 μM), either in the presence or in the absence of Y-27632 (10 μM) or U0126 (10 μM), for 16 h. Cells were pulsed with bromodeoxyuridine (BrdU; 10 μM) for 2 h prior to harvesting with trypsin. Cells were fixed with ice-cold 70% ethanol for 20 min at room temperature and then treated with 3 N HCl containing 0.5% Triton X-100 for 20 min. Residual acid was neutralized by incubating the cell suspension with 0.1 M sodium borate (pH 8.5) for 2 min at room temperature. Cells were then incubated with anti-BrdU monoclonal antibody (555627; BD Biosciences Pharmingen) diluted 1:200 for 20 min, followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (554001; BD Biosciences Pharmingen) for 20 min. The cell suspension was incubated with propidium iodide (5 μg/ml) for 30 min and then analyzed with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson).

siRNA transfection. Gene silencing was achieved by transient transfection of small interfering RNA (siRNA) duplexes (Dharmacon, Inc., Lafayette, CO). siRNA duplexes against mouse cyclin A (*CCNA2*; siGenome duplexes 2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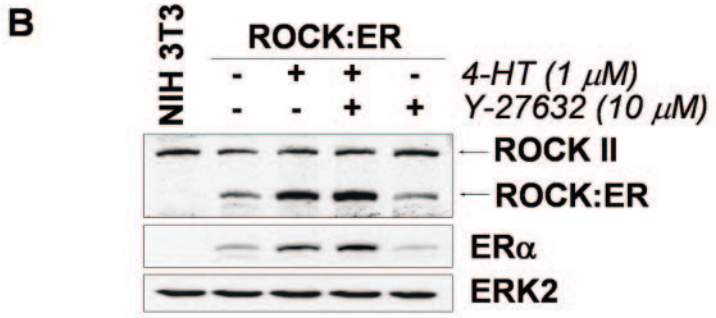
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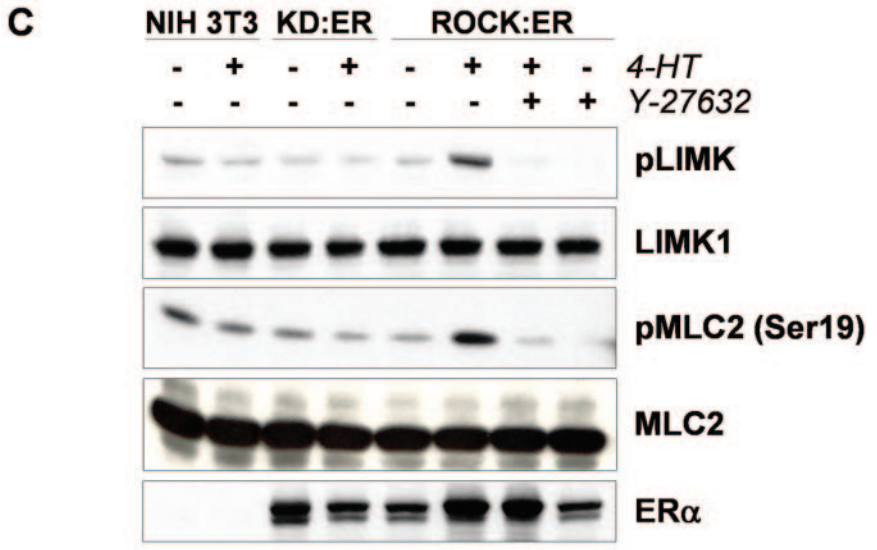
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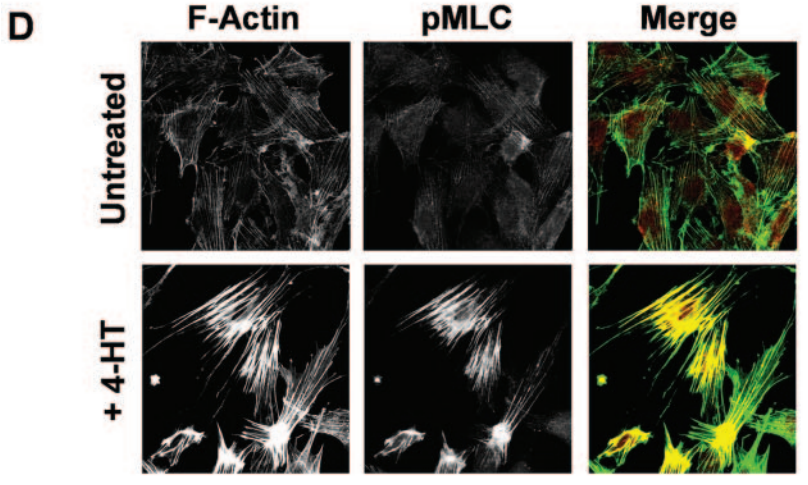
D
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[D-040393-02] and 3 [D-040393-03]), cyclin D1 (*CCND1*; siGenome duplexes 3 [D-042441-03] and 4 [D-042441-04]), p27 (*CDKN1B*; siGenome duplexes 3 [D-040178-02] and 4 [D-040178-04]), lamin A/C (D-001050-01; Dharmacon), LIMK 1 (siGenome duplexes 2 [D-043923-02] and 3 [D-043923-03]), LIMK 2 (siGenome duplexes 1 [D-043932-01] and 2 [D-043932-02]), and nontargeting control 1 (D-001210-01) were used. In brief, NIH 3T3 ROCK-ER cells were plated at 1.2×10^5 cells per well of a six-well plate in antibiotic-free medium. Cells were transfected the following day with 4 μl of Lipofectamine 2000 (Invitrogen, Paisley, United Kingdom) and 50 nM siRNA duplexes per well according to the manufacturer's instructions. After 6 h, 20% DCS was added to give a final serum concentration of 10%. siRNA complexes were removed 16 h later, and fresh 10% DCS was added. Twenty-four hours later, cells were trypsinized and plated at 0.5×10^6 cells per 10-cm plate in 10% DCS. The following day, cells were serum starved and then treated with or without 4-HT (1 μM) for 16 h. Cells were harvested as described above.

In vitro cyclin E-CDK2 kinase assays. Cells were washed twice in ice-cold PBS and lysed in ELB+ buffer (250 mM NaCl, 50 mM HEPES [pH 7.0], 5 mM EDTA, 10 mM β-glycerol phosphate, 10 mM NaF, 1 mM sodium vanadate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Equal amounts of cell lysate (400 μg in 0.5 ml of ELB+ buffer) were incubated with 3 μg of anti-cyclin E (07-687; BD Biosciences) bound to 40 μl of protein A-agarose beads (Upstate) for 90 min at 4°C. Immunocomplexes were washed twice in ELB+, once in 50 mM HEPES (pH 7.4), and once in kinase buffer (50 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 10 mM β-glycerol phosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The washed immunoprecipitates were resuspended in 40 μl of kinase buffer containing 2 μg of histone H1 (Upstate), 50 μM ATP, and 5 μCi of [γ-^{32}P]ATP and incubated at 30°C for 15 min. Kinase reactions were stopped by addition of 10 μl of 6× Laemmli buffer. After SDS-polyacrylamide gel electrophoresis and transfer, ^{32}P-labeled histone H1 was visualized by exposure to Biomax film (Kodak). Membranes were subsequently blotted with antibodies against CDK2 (06-505; Upstate) and p21 (sc-397G; Santa Cruz).

RESULTS

ROCK activation promotes G_1/S cell cycle progression. To determine whether the selective activation of ROCK was sufficient to induce cell cycle progression, we utilized conditionally activated ROCK-ER, which was generated by fusing ROCK II amino acids 5 to 553, comprising the catalytic kinase domain and a portion of the coiled-coil domain, to enhanced green fluorescent protein and the hormone-binding domain of the estrogen receptor (Fig. 1A) (17). We previously used ROCK-ER to examine the role of ROCK in keratinocyte differentiation (46), cell invasiveness (18, 70), and apoptotic nuclear breakdown (16). NIH 3T3 mouse fibroblasts, which have a biallelic deletion of the *INK4a* locus resulting in loss of p16 expression, were transduced with retrovirus expressing ROCK-ER (Fig. 1B). When cell lysates were blotted with ROCK II antibody, there was no detectable ROCK-ER protein in untransduced NIH 3T3 cells and approximately equal levels of ROCK II and ROCK-ER in untreated ROCK-ER-expressing cells. Consistent with previous results (17), treatment with the estrogen analogue 4-HT resulted in a roughly twofold increase in ROCK-ER expression, which was not affected by the ROCK inhibitor Y-27632. Western blotting with anti-estrogen receptor antibody (ERα) confirmed the identity of the ROCK-ER fusion protein. To determine the functional effects of ROCK activation, cells that were untransduced or expressing either ROCK-ER or a kinase-dead version (K121G) called KD-ER were treated with 4-HT and the effects on the phosphorylation of ROCK substrates were determined (Fig. 1C). Treatment with 1 μM 4-HT for 16 h had no effect in parental NIH 3T3 or KD-ER-expressing cells, but ROCK-ER-expressing cells had increased levels of phosphorylated LIMK1 (Thr508) and/or LIMK2 (Thr505) and phosphorylated regulatory myosin light chain (MLC2, Ser19). Coadministration of the ROCK-selective inhibitor Y-27632 (75) blocked the 4-HT-induced increase in phospho-LIMK and phospho-MLC, which indicated that these events were due to ROCK-ER activation by 4-HT and is in agreement with the lack of effect in 4-HT-treated parental NIH 3T3 and KD-ER-expressing cells. Western blotting with ERα antibody confirmed the expression of KD-ER and ROCK-ER. When the effects on the actin-myosin cytoskeleton were examined, it was clear that ROCK-ER activation by 4-HT resulted in dramatic increases in F-actin, which colocalized with phospho-MLC in prominent contractile stress fibers (Fig. 1D). Treatment of parental NIH 3T3 cells or KD-ER-expressing cells with 4-HT had no effect, while the induction of actin stress fibers in ROCK-ER-expressing cells could be blocked by Y-27632 coadministration (17), again confirming that these events were dependent upon ROCK-ER catalytic activity.

In order to determine whether ROCK activity was sufficient to induce cell cycle progression, ROCK-ER-expressing NIH 3T3 cells were serum starved and then either left untreated or treated with 1 μM 4-HT alone or in combination with 10 μM Y-27632 for 16 h prior to a 2-h pulse with BrdU, followed by fixation, staining with propidium iodide, and FACS analysis (Fig. 2A, percentage of S-phase cells for each condition indicated). Mean data from three experiments revealed that only

FIG. 1. ROCK-ER activation induces phosphorylation of ROCK substrates and stress fiber formation. (A) Schematic representation of ROCK II showing functional domains (PH = pleckstrin homology domain, CRD = cysteine-rich domain). Conditionally regulated ROCK-ER was generated by fusing amino acids 5 to 553 of ROCK II between the enhanced green fluorescent protein (EGFP) and the estrogen receptor hormone binding domain (hbER). KD-ER was created by changing lysine 121 to glycine [ROCK (K121G)]. (B) NIH 3T3 cells were transduced with retrovirus encoding the conditionally active ROCK-ER fusion proteins. Serum-starved parental NIH 3T3 or ROCK-ER-expressing cells were left untreated or treated with 1 μM 4-HT for 16 h as indicated, either in the presence or in the absence of ROCK inhibitor Y-27632 (10 μM). ROCK II and ROCK-ER protein levels were determined by Western blotting with an antibody against a common epitope. Fusion protein expression was confirmed by blotting with ERα antibody. Results of a representative experiment of three determinations with similar results are shown. (C) NIH 3T3 cells were transduced with retrovirus encoding the conditionally active ROCK-ER or kinase-dead KD-ER fusion protein. Serum-starved parental NIH 3T3, KD-ER-expressing, or ROCK-ER-expressing cells were either left untreated or treated with 1 μM 4-HT for 16 h as indicated, either in the presence or in the absence of ROCK inhibitor Y-27632 (10 μM). Cells were lysed, and protein phosphorylation status was determined with antibodies against phospho-MLC2 (Ser19) and phospho-LIMK1/LIMK2 (antibody recognizing a common epitope at Thr508 and Thr505, respectively). Fusion protein expression was confirmed by blotting with ERα antibody. Results of a representative experiment of three determinations with similar results are shown. (D) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated or treated with 1 μM 4-HT for 16 h and then fixed and stained for F-actin (green) and phospho-Ser19 MLC (red). Results of a representative experiment of two determinations with similar results are shown.

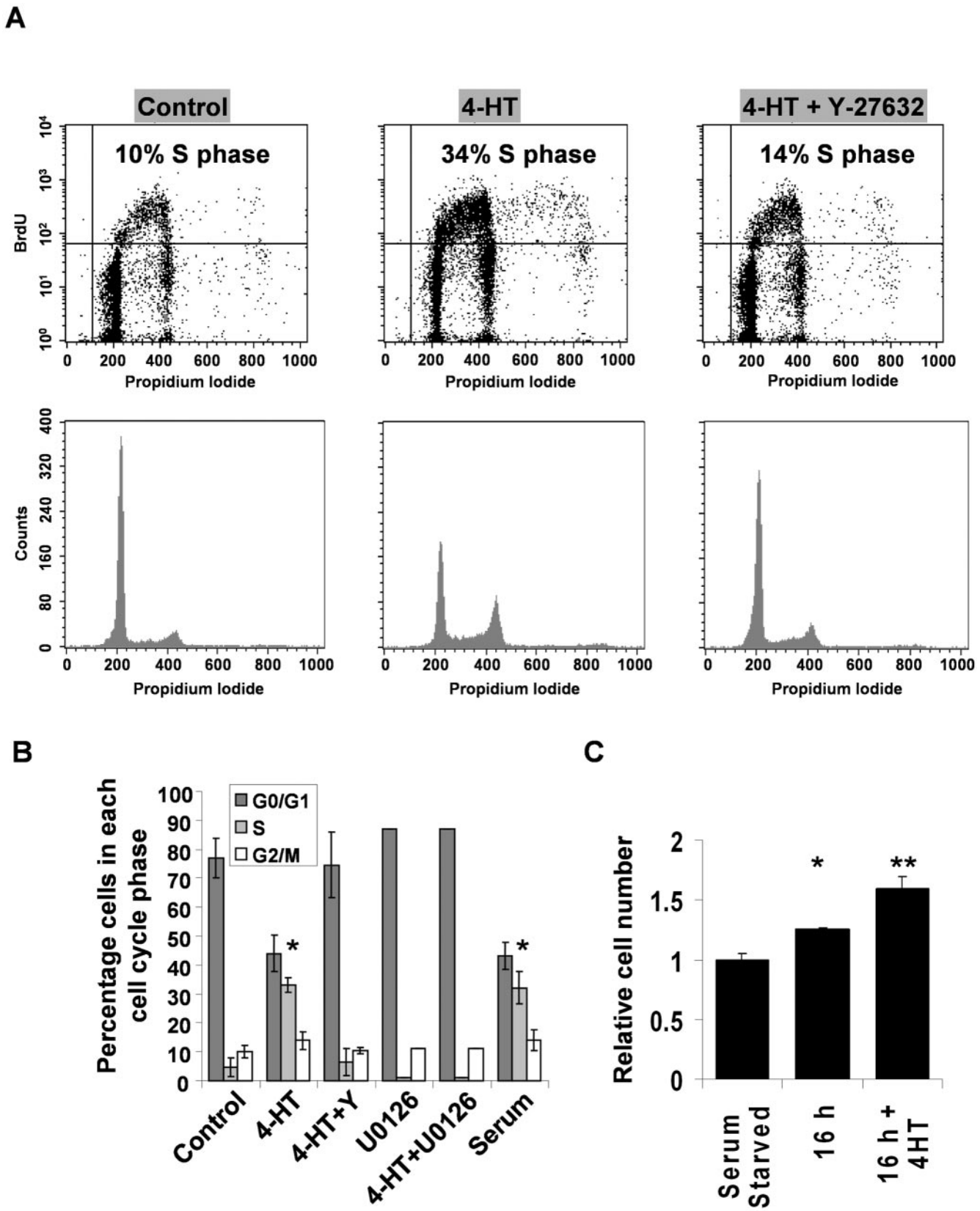

FIG. 2. ROCK-ER activation promotes S-phase entry and increased cell number. (A) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated (Control) or treated with 1 μM 4-HT for 18 h either in the presence or in the absence of ROCK inhibitor Y-27632 (10 μM). Cells were pulsed with 10 μM BrdU for 2 h prior to harvest and then collected by trypsinization. Cells were fixed, stained with anti-BrdU

5% ± 3% of the serum-starved control cells were BrdU positive, indicating passage through the DNA-synthetic S phase, whereas a statistically significant 33% ± 3% of the 4-HT-treated cells were in S phase, comparable to the significant 32% ± 5% S-phase cells observed following treatment with medium containing 10% serum (Fig. 2B). Passage through to the G_2/M phases was also higher in 4-HT-treated cells (14% ± 3% for 4-HT-treated cells versus 10% ± 2% for serum-starved cells). Consistent with the previous results for 4-HT-induced phosphorylation of ROCK substrates (Fig. 1C), 4-HT-stimulated cell cycle progression was blocked by Y-27632 (Fig. 2B). In addition, coadministration of 4-HT with the MEK inhibitor U0126 (10 μM) reduced the percentage of BrdU-positive cells to the levels observed in cells treated with U0126 alone, indicating that the ROCK-stimulated cell cycle progression was dependent on MAPK signaling.

We also wished to determine whether ROCK-ER activation would promote cell cycle progression through to the completion of cytokinesis. ROCK-ER-expressing cells were serum starved for 24 h and then either counted in triplicate or allowed to proceed for another 16 h without or with the addition of 4-HT. Although the untreated group did have a significant 25% increase in cell number, activation of ROCK-ER led to a 60% increase in cell number during the same period, which was significantly greater than either of the untreated groups (Fig. 2C). Therefore, in addition to the increased proportion of cells with G_2/M DNA content in Fig. 2B, the increase in cell number indicates that ROCK activation also promotes completion of cell division.

ROCK influence on cell cycle regulatory proteins. Given that ROCK activation stimulated cell cycle progression, we next examined how ROCK influenced the levels of key cell cycle regulatory proteins. Consistent with previous results (Fig. 1C), 4-HT treatment of KD-ER-expressing cells did not alter LIMK phosphorylation, whereas ROCK-ER-expressing cells responded with increased LIMK phosphorylation that could be blocked by coadministration of Y-27632 (Fig. 3A). Similarly, 4-HT did not alter the expression of cell cycle regulatory proteins in KD-ER-expressing cells, but cyclin A2 (hereafter called cyclin A), cyclin D1, and p21 levels increased while p27 levels decreased in 4-HT-treated ROCK-ER-expressing cells. In addition, coadministration of Y-27632 blocked the 4-HT-induced effects while Y-27632 treatment alone reduced basal levels of phospho-LIMK, cyclin A, cyclin D1, and p21. No change in the expression of cyclin E (Fig. 3A), $p18^{INK4C}$, CDK2, CDK4, or CDK6 (data not shown) was observed under any condition, while $p15^{INK4B}$ and cyclins A1, D2, and D3 were undetectable in all treatment groups (data not shown). Given the importance of cyclin D1 nuclear localization for the stimulation of cell cycle progression (20), we examined cyclin D1 localization following 4-HT treatment. Similar to total cyclin D1 levels observed by Western blotting (Fig. 3A), immunofluorescence analysis revealed that untreated serum-starved cells had little cyclin D1 (Fig. 3B). Treatment with 4-HT significantly increased cyclin D1 expression, which accumulated within cell nuclei. Coadministration of 4-HT with Y-27632 inhibited both cyclin D1 expression and nuclear accumulation. We also compared the time course of ROCK-ER activation with the induction of cyclin D1 and found that although strong LIMK phosphorylation was observed at all time points following 4-HT treatment, expression of cyclin D1 lagged and was not evident until 16 h of treatment (Fig. 3C).

ROCK induction of cyclin D1 expression via Ras/MAPK. Arguably, the primary function of ROCK is to regulate cellular contractility by influencing actin-myosin cytoskeletal structures. Therefore, we tested whether disrupting F-actin would affect ROCK-ER regulation of cyclin A and D1 expression. Cells were treated with CCD, which caps actin filaments and stimulates ATP hydrolysis on G-actin (62); SWA, which sequesters G-actin as dimers (8); LTB, which sequesters G-actin monomers (69); or Jasp, which disrupts F-actin and induces polymerization of G-actin into amorphous masses (9). Each of these treatments did not affect cyclin E expression or LIMK phosphorylation in response to 4-HT activation of ROCK-ER; however, cyclin D1 expression was eliminated by the actin-disrupting compounds (Fig. 4A). The effects on cyclin A expression were mixed; in each case, basal expression was elevated, which could be further increased by ROCK-ER activation in CCD- or LTB-treated cells. In SWA- and Jasp-treated cells, cyclin A levels could not be increased further by ROCK-ER activation, suggesting that maximal expression had been achieved. Since it has been shown that the Ras/MAPK pathway regulates cyclin D1 expression (12), we examined Ras activation in ROCK-ER-expressing cells by using a pull-down assay with the Ras-binding domain of Raf-1. ROCK-ER activation with 4-HT led to a significant ($P < 0.05$) twofold increase in Ras-GTP levels, relative to untreated cells, which could be blocked by coadministration with Y-27632 (Fig. 4B). Treatment of cells with CCD to disrupt actin structures increased basal Ras activation and led to only an ~25% increase

antibody and propidium iodide, and then analyzed by flow cytometry. Top panels show BrdU staining versus propidium iodide staining to quantify S-phase cells, and bottom panels show cell numbers versus propidium iodide staining to quantify DNA content. (B) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated (Control) or treated with 1 μM 4-HT for 18 h, either in the presence or in the absence of Y-27632 (10 μM) or U0126 (10 μM). Cells were pulsed with 10 μM BrdU for 2 h prior to harvest and then collected by trypsinization. Cells were fixed and stained with anti-BrdU antibody and propidium iodide, flow cytometry was performed, and the cell cycle distribution was analyzed. Mean values ± the standard error of the mean from three repetitions are shown, except for U0126 treatment groups, where mean values from duplicate determinations are reported. Statistical significance was determined by Student's *t* test, and an asterisk indicates significant difference ($P < 0.05$) from serum-starved condition. (C) Three independent pools of ROCK-ER-expressing NIH 3T3 cells were serum starved for 24 h and then either left untreated or treated with 1 μM 4-HT for 16 h. The numbers of cells after initial serum starvation and after the additional 16 h without or with 4-HT were determined. Mean values ± the standard error of the mean, normalized to serum-starved cells, are indicated. Statistical significance was determined by Student's *t* test, and an asterisk indicates significant difference ($P < 0.05$) from the initial serum-starved condition while a double asterisk indicates significant difference ($P < 0.05$) from both serum-starved conditions. Results of a representative experiment of two determinations with similar results are shown.

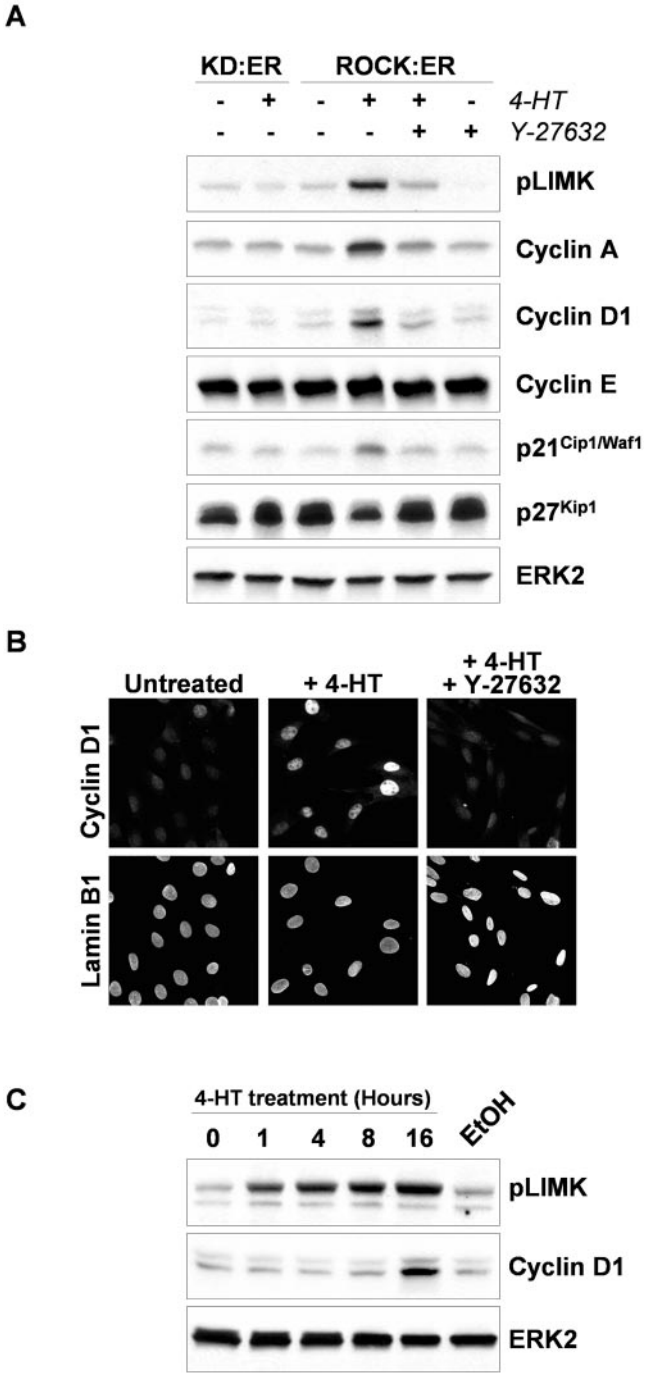

FIG. 3. Expression of cell cycle regulators following ROCK-ER activation. (A) Serum-starved, KD-ER- and ROCK-ER-expressing NIH 3T3 cells were either left untreated or treated with 1 μM 4-HT for 16 h, either in the presence or in the absence of 10 μM Y-27632. Cell lysates were Western blotted for phospho-LIMK1/2, cyclin A, cyclin D1, cyclin E, p21, and p27. Equal protein loading was confirmed by blotting with ERK2 antibody. Results of a representative experiment of three determinations with similar results are shown. (B) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated or treated with 1 μM 4-HT, either in the presence or in the absence of 10 μM Y-27632, for 16 h. Coverslips were fixed, permeabilized, and then stained with anti-cyclin D1 and anti-lamin B1 antibodies. Results of a representative experiment of two determinations with similar results are shown. (C) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were treated with 1 μM 4-HT for the times indicated. Lysates were analyzed by immunoblotting with antibodies against cyclin D1, phospho-LIMK1/2, and ERK2. Results of a representative experiment of three determinations with similar results are shown. EtOH, ethanol.

induced by ROCK-ER that was not statistically significant, suggesting that an intact actin cytoskeleton was required for robust Ras activation by ROCK, consistent with previous reports (83).

In order to determine whether cytoskeletal integrity was necessary for ROCK-ER induction of cell cycle progression, ROCK-ER-expressing NIH 3T3 cells were serum starved and then either left untreated or treated with 1 μM 4-HT alone or in combination with CCD or Jasp for 16 h prior to a 2-h pulse with BrdU, followed by fixation, staining with propidium iodide, and FACS analysis. Mean data from three experiments revealed that only 2% ± 1% of the serum-starved controls cells were BrdU positive, indicating passage through the DNA-synthetic S phase, whereas 35% ± 4% of the 4-HT-treated cells were in S phase (Fig. 4C). Treatment with CCD or Jasp alone did not affect the proportion of S-phase cells but did result in accumulation of the G_2/M population, likely because of inhibition of cytokinesis. Administration of CCD or Jasp resulted in significant reductions in the percentage of S-phase cells induced by 4-HT to 9% ± 3% and 6% ± 5%, respectively. These results indicate that the actin cytoskeleton is the principal mediator of ROCK influence on the cell cycle, consistent with the key contribution of the cytoskeleton to cyclin D1 induction by ROCK (Fig. 4A).

Given the increased Ras-GTP level following ROCK activation, the sensitivity of both cyclin D1 elevation (Fig. 4A) and Ras activation (Fig. 4B) to CCD treatment, and the inhibition of ROCK-ER-stimulated cell cycle progression by the MEK inhibitor U0126 (Fig. 2B), one possibility was that the regulation of some cell cycle proteins by ROCK-ER might be via the MAPK pathway. We treated ROCK-ER-expressing cells with 4-HT alone in combination with Y-27632 or with U0126 and then Western blotted cell extracts (Fig. 4D). Although the 4-HT induction of LIMK phosphorylation was sensitive to Y-27632, U0126 had no effect. However, inhibition of MEK resulted in inhibition of ERK phosphorylation and loss of cyclin D1 and p21 induction. In contrast, the induction of cyclin A and the decrease in p27 levels were unaffected by U0126. These data are consistent with the hypothesis that ROCK, acting via actin cytoskeletal structures, activates Ras and the MAPK pathway, leading to the elevation of cyclin D1 and p21 expression. Cyclin A and p27 are apparently regulated through MAPK-independent mechanisms, which also are independent of cell cycle progression since U0126 blocked entry into S phase (Fig. 2B) without affecting the regulation of cyclin A or p27 (Fig. 4D).

ROCK regulation of cyclin expression is independent of focal adhesions. In addition to regulating actin stress fibers, RhoA signals through ROCK to promote the formation of integrin-containing focal adhesions, leading to increased substrate adherence (41, 54, 75). Therefore, we wished to determine whether the effects of ROCK-ER activation were mediated through focal adhesion signaling. ROCK-ER-expressing

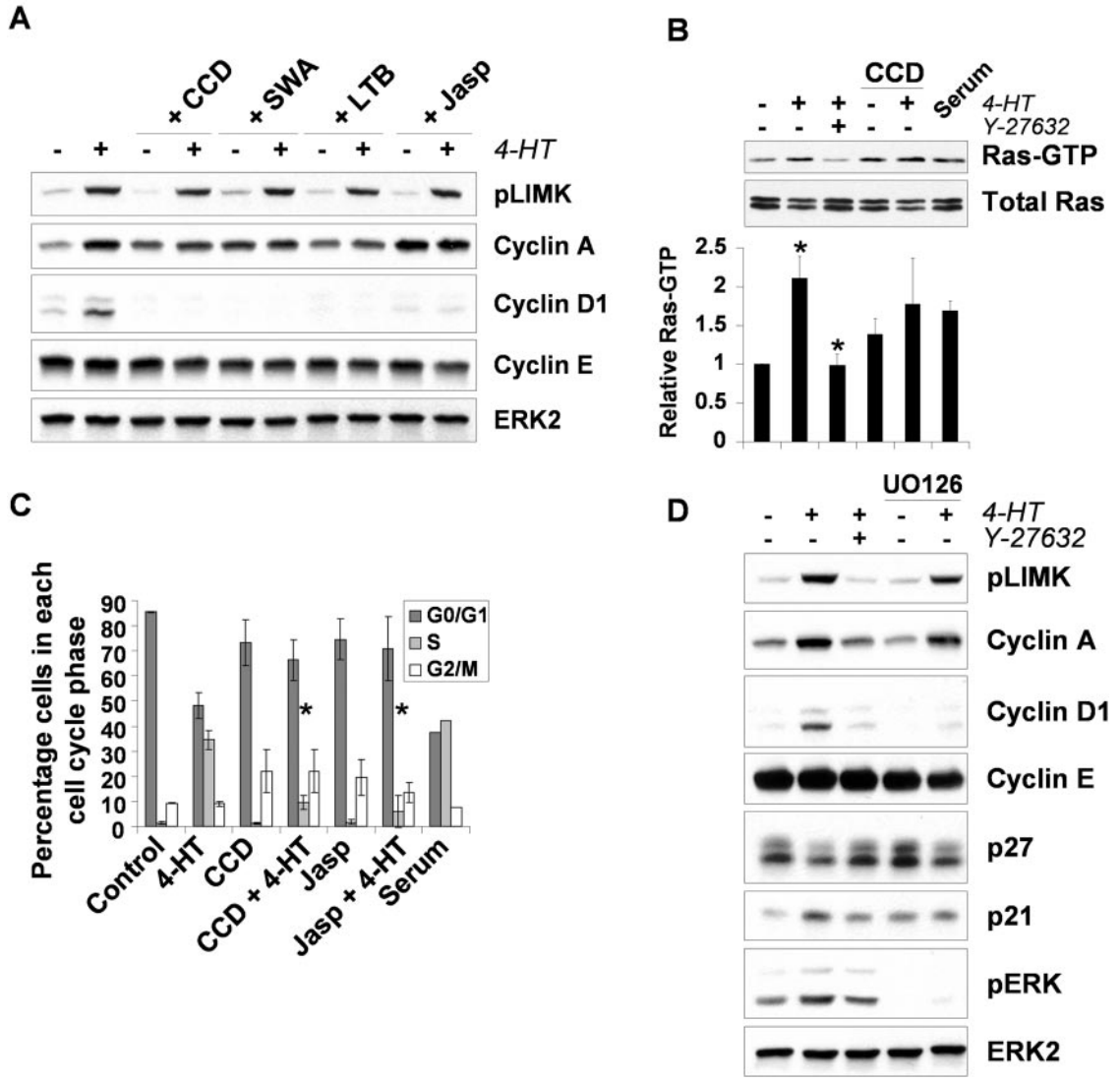

FIG. 4. ROCK-ER induction of cyclin D1 by actin-mediated Ras activation. (A) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were left untreated or treated with 1 μM 4-HT for 16 h, either in the presence or in the absence of 1 μM CCD, 0.05 μM SWA, 0.5 μM LTB, or 0.5 μM Jasp. Whole-cell lysates were analyzed by Western blotting for cyclin A, cyclin D1, cyclin E, and phospho-LIMK1/2. Blotting with ERK2 antibody was used to confirm equal protein loading. Results of a representative experiment of three determinations with similar results are shown. (B) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated or treated with 1 μM 4-HT or 5% serum as indicated, in the presence or absence of 10 μM Y-27632 or 1 μM CCD, for 16 h. Active Ras-GTP was purified with glutathione *S*-transferase–Raf-1 RBD agarose beads. GTP-loaded Ras and total Ras were detected by Western blotting. Quantitation is averaged results from three separate experiments; average values ± the standard error of the mean are shown. An asterisk indicates a significant difference, as determined by Student's *t* test, at $P < 0.05$. (C) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated (Control) or treated with 1 μM 4-HT for 18 h either in the presence or in the absence of 1 μM CCD or 0.5 μM Jasp. Cells were pulsed with 10 μM BrdU for 2 h prior to harvest and then collected by trypsinization. Cells were fixed and stained with anti-BrdU antibody and propidium iodide, flow cytometry was performed, and the cell cycle distribution was analyzed. Mean values ± the standard error of the mean from three repetitions are shown, except for the serum treatment group, where mean values from duplicate determinations are reported. Statistical significance was determined by Student's *t* test, and an asterisk indicates significant difference ($P < 0.05$) from the 4-HT-treated condition. (D) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated or treated with 1 μM 4-HT for 16 h, either in the presence or in the absence of 10 μM Y-27632 or 10 μM U0126. Cell lysates were immunoblotted for phospho-LIMK1/2, cyclin A, cyclin D1, cyclin E, p27, p21, and phospho-ERK. Equal protein loading was confirmed by blotting with ERK2 antibody. Results of a representative experiment of three determinations with similar results are shown.

cells were plated on untreated plastic tissue culture dishes as before, on fibronectin-coated dishes to promote the formation of integrin-containing focal adhesions, or on PDL-coated dishes to allow adhesion without integrin clustering. Activation of ROCK-ER with 4-HT consistently led to LIMK phosphorylation, increased cyclin D1 expression, and increased ERK and MEK phosphorylation in cells plated on each substrate (Fig. 5A). In addition, cyclin D1 induction and ERK phosphorylation were blocked by U0126 under all three conditions, indicating that these events were mediated by the MAPK path-

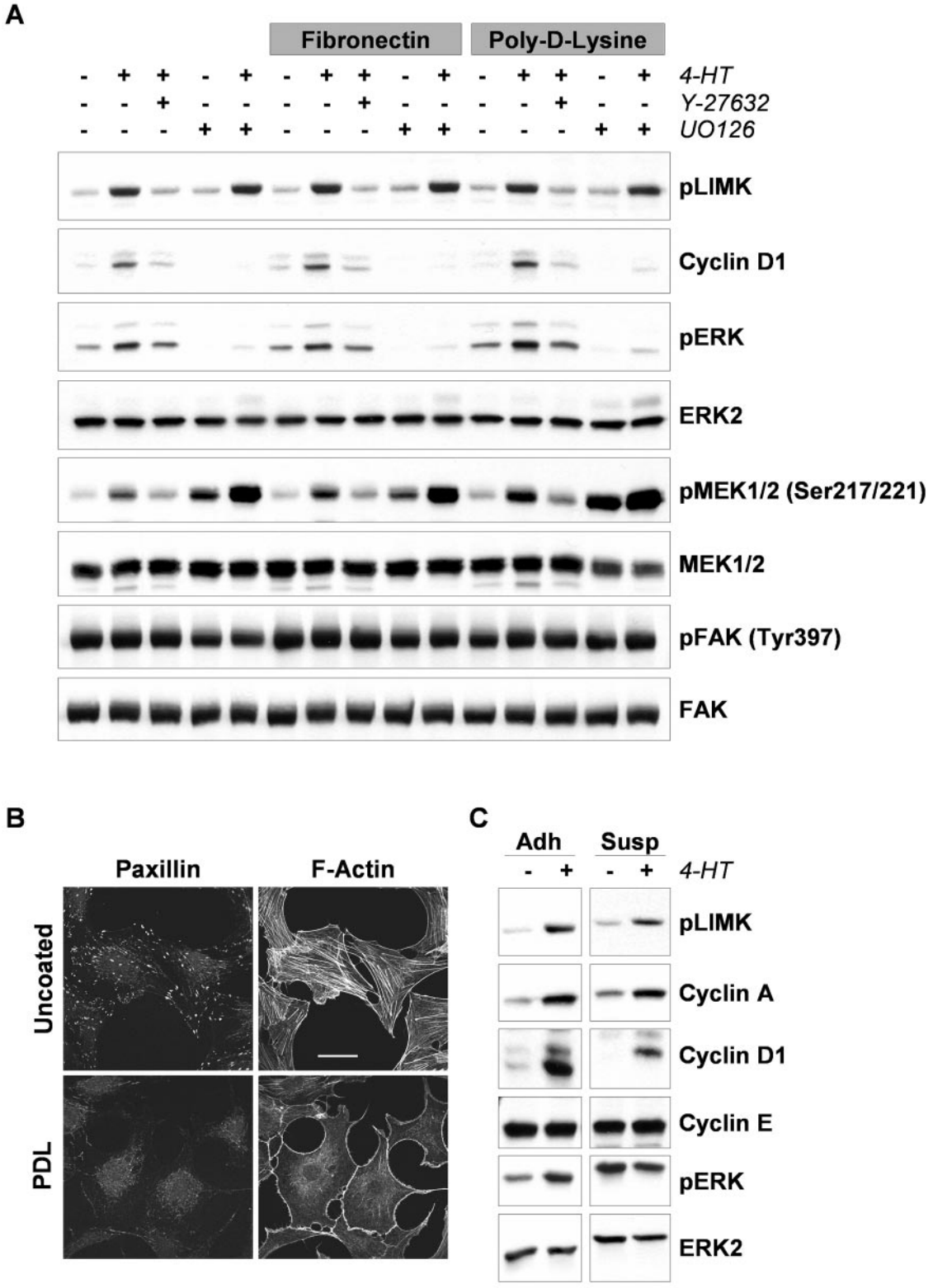

FIG. 5. Adhesion and integrin signaling are not required for ROCK-induced cyclin D1 expression. (A) ROCK-ER-expressing NIH 3T3 cells were plated on uncoated tissue culture dishes or dishes coated with either fibronectin or PDL. Subconfluent cells were serum starved and then left untreated or treated with 1 μM 4-HT for 16 h, either in the presence or in the absence of 10 μM Y-27632 or 10 μM U0126. Cell lysates were Western blotted for phospho-LIMK1/2, cyclin D1, phospho-ERK, ERK2, phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-FAK (Tyr397), and FAK. Results of a representative experiment of three determinations with similar results are shown. (B) ROCK-ER-expressing NIH 3T3 cells were plated on uncoated glass coverslips or PDL-coated coverslips. Subconfluent cells were serum starved overnight and then left in serum-free medium for an additional 16 h. After fixation and permeabilization, cells were stained with anti-paxillin antibody to visualize focal adhesions and with

way. Treatment with U0126, which works by inhibiting MEK catalytic activity (19), blocked ERK phosphorylation but increased basal MEK phosphorylation, which could be further elevated by ROCK-ER activation. These findings are consistent with previous studies that showed that treatment with MEK inhibitors led to increased MEK phosphorylation (5, 19, 31, 76). One possible explanation is that although these inhibitors block MEK activity, they can actually stimulate Raf activity (2, 82), likely by reducing the effects of ERK-induced negative feedback mechanisms. In contrast, there was no increase in phosphorylation of FAK on the Src-kinase binding site Tyr397. In parallel, cells plated on uncoated coverslips or PDL-coated coverslips were serum starved as in Fig. 5A and then stained for paxillin localization to visualize focal adhesions and with phalloidin for F-actin structures. As shown in Fig. 5B, cells plated on uncoated coverslips had numerous intense paxillin-containing focal adhesions and prominent stress fibers. In contrast, cells on PDL had dramatically fewer focal adhesions and rare small stress fibers, the predominant cytoskeletal structure being cortical actin. These data suggest that ROCK-ER stimulation of MAPK and cyclin D1 expression is independent of integrin signaling.

We extended these findings by plating ROCK-ER-expressing cells on either untreated plastic tissue culture dishes or dishes that had been coated with poly-HEMA, which does not permit adhesion. Adherent or suspended cells treated with 4-HT to activate ROCK-ER had increased LIMK phosphorylation and cyclin A and D1 expression, although cyclin D1 induction was slightly attenuated in suspension cells (Fig. 5C). Interestingly, phospho-ERK levels were elevated in untreated cells in suspension, consistent with previous results reported for NIH 3T3 cells (84), primary mouse embryo fibroblasts (89), ARLJ301-3 rat liver cells (28), MDCK cells (11, 15), and growth-arrested MCF-10A mammary epithelial cells (14). However, cyclin D1 was not induced unless ROCK-ER was activated with 4-HT, indicating that ROCK-ER regulation of cyclin D1 has both MAPK-dependent and -independent components and that the influence of ROCK on the expression of cell cycle regulatory proteins does not require adhesion.

ROCK regulates cell cycle proteins via independent pathways. In order to determine whether the changes in cyclin A, cyclin D1, and p27 are independent events, we used siRNA to knock down the expression of each protein and examined the effects of ROCK-ER activation on the remaining proteins. We were particularly interested in the relationship between cyclin A induction and p27 down-regulation, given that cyclin A-CDK2 has been reported to phosphorylate p27 on T187, leading to its ubiquitylation and degradation (47), and that cyclin A also has been proposed to regulate p27 via a noncatalytic mechanism (88). We were only able to partially knock down cyclin A with two independent siRNA duplexes but observed no significant effects on LIMK phosphorylation or on cyclin D1 and p27 levels following 4-HT activation of ROCK-ER (Fig. 6A). Transfection with control siRNA or lamin A/C duplexes also had no effect on cyclin D1, cyclin A, or p27 regulation in response to 4-HT activation of ROCK-ER (Fig. 6A to C). Similarly, the efficient knockdown achieved with either cyclin D1 siRNA duplex had no effect on LIMK phosphorylation or cyclin A induction following ROCK-ER activation (Fig. 6B), consistent with the lack of effect on cyclin A induction by reduced cyclin D1 expression following treatment with U0126 (Fig. 4D). Finally, knockdown of p27 did not change ROCK-ER-induced LIMK phosphorylation or cyclin A and cyclin D1 elevation (Fig. 6C). These data indicate that cyclin A, cyclin D1, and p27 are each independently regulated by ROCK.

Transcription of the *cyclin A2* gene has been shown to be regulated largely by "pocket proteins" such as Rb or p107, which are phosphorylated by cyclin D1- and cyclin E-associated CDKs (6). However, the actin cytoskeleton has also been shown to be an important regulator of cyclin A expression, independent of pocket proteins (3, 7). Given that cyclin A induction was still observed following MEK inhibitor treatment (Fig. 4D) and after cyclin D1 knockdown (Fig. 6B), we examined the phosphorylation of p107 in response to ROCK activation. As shown in Fig. 7A, treatment with 4-HT increased phospho-LIMK and led to a reduced-mobility form of p107, indicative of increased phosphorylation. Efficient knockdown of cyclin D1 still allowed 4-HT induction of LIMK phosphorylation but inhibited p107 phosphorylation. Consistent with these findings, treatment with the MEK inhibitor U0126 blocked 4-HT-induced elevation of cyclin D1 levels and p107 phosphorylation (Fig. 7B). We next determined whether knockdown of cyclin D1 led to changes in cyclin E-CDK2 activity. As also shown in Fig. 6B and 7A, knockdown of cyclin D1 did not affect 4-HT-induced LIMK phosphorylation; however, cyclin E-associated CDK2 activity was effectively inhibited in both siRNA treatment groups (Fig. 7C). Given that one of the primary functions of cyclin D1-CDK4 and cyclin D1-CDK6 complexes is to titrate p21 and p27 CDKIs away from cyclin E-CDK2 (39), inhibition of cyclin E-associated CDK2 activity would be an expected outcome. Consistent with a role in CDKI titration, increased p21 was associated with cyclin E complexes following cyclin D1 siRNA and 4-HT treatment. It is possible, however, that additional factors contribute to the decreased CDK2 activity observed following cyclin D1 knockdown. These results are consistent with those presented in Fig. 4D and 6B, revealing that ROCK induction of cyclin A is not only independent of cyclin D1 but also essentially independent of pocket protein phosphorylation.

LIMK mediates ROCK induction of cyclin A. Although ROCK phosphorylates a number of proteins (55), the only ROCK substrates previously implicated in cell cycle regulation were the LIMKs (58). Therefore, we used siRNA to examine the contribution of LIMK1 and LIMK2 to ROCK-ER-medi-

phalloidin to visualize F-actin structures. The scale bar represents 35 μm. Results of a representative experiment of four determinations with similar results are shown. (C) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were trypsinized, resuspended in serum-free DMEM, and replated on either uncoated tissue culture dishes (adherent [Adh]) or poly-HEMA-treated dishes (suspension [Susp]). Cells were left untreated or treated with 1 μM 4-HT for 16 h. Cell lysates were Western blotted for phospho-LIMK1/2, cyclin A, cyclin D1, cyclin E, phospho-ERK, and ERK2. Results of a representative experiment of three determinations with similar results are shown.

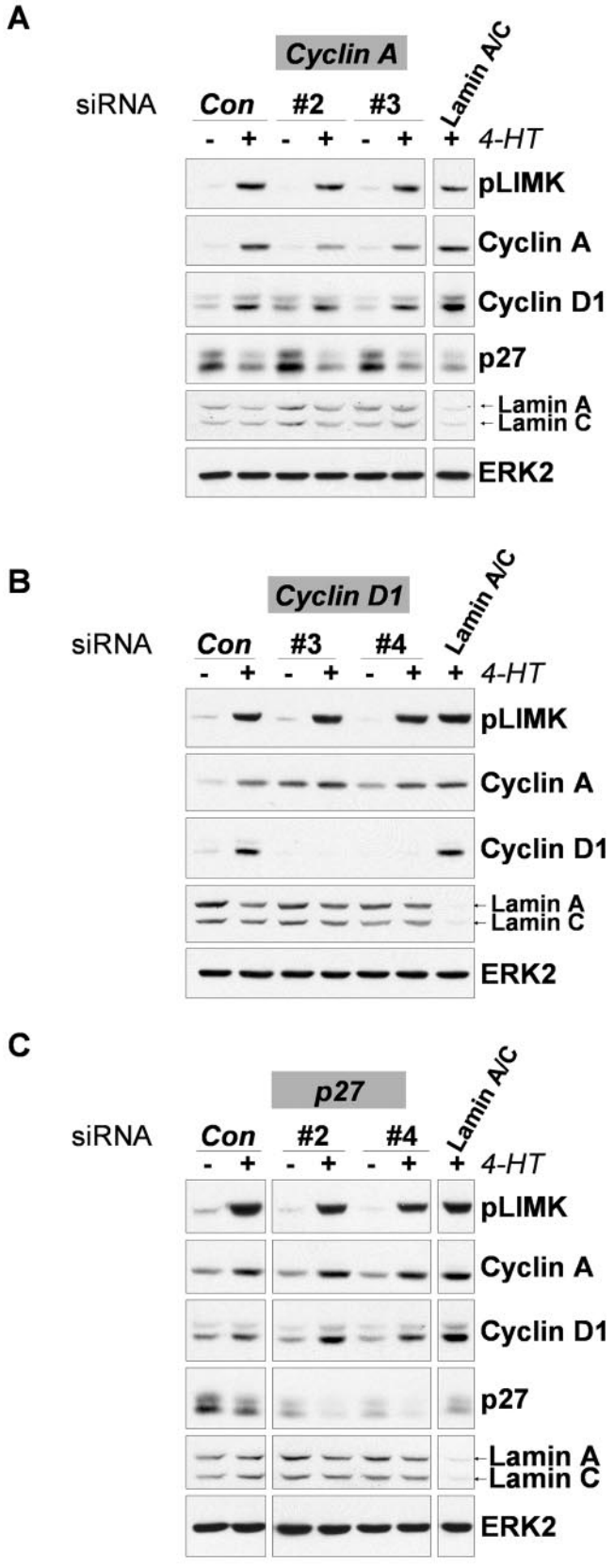

FIG. 6. Independent effects of ROCK-ER on cyclin A, cyclin D1, and p27 levels. ROCK-ER-expressing NIH 3T3 cells were transfected with control nontargeting (*Con*) siRNA or siRNA duplexes targeting *Lamin A/C*, *Cyclin A* (A), *Cyclin D1* (B), or *p27* (C). At 24 h posttransfection, cells were trypsinized and replated on plastic tissue culture dishes. Subconfluent cells were serum starved and then either left untreated or treated with 1 μM 4-HT for 16 h. Whole-cell lysates were immunoblotted for phospho-LIMK, cyclin A, cyclin D1, p27, and lamin A/C. Equal protein loading was confirmed by blotting with ERK2 antibody. Results of representative experiments of three or four determinations with similar results are shown.

ated changes in the expression of cell cycle regulatory proteins. The profound knockdown of LIMK1 only slightly affected the level of phospho-LIMK observed following ROCK-ER activation with 4-HT, suggesting that LIMK2 is the major protein detected by the phospho-LIMK antibody (Fig. 8A). Consistent with this interpretation, knockdown of LIMK2 significantly reduced the phospho-LIMK observed after 4-HT treatment, and LIMK1 expression was significantly lower than LIMK2 expression in Western blot assays with all of the antibodies we have tested (data not shown). LIMK1 knockdown did not affect the induction of cyclin A and cyclin D1 or the decrease in p27 following ROCK-ER activation. In contrast, knockdown of LIMK2 reduced the cyclin A induction by ROCK-ER activation. Cyclin D1 levels were elevated when LIMK2 was knocked down and could not be raised further by ROCK-ER activation, consistent with the reported function of LIMK as a repressor of cyclin D1 transcriptional induction by MAPK-independent signaling pathways (58). Similar to LIMK1, knockdown of LIMK2 did not affect ROCK-ER-induced reduction in p27 protein levels. The combined knockdown of LIMK1 and LIMK2 had the same results as knockdown of LIMK2 alone, in that ROCK-ER induction of cyclin A expression was reduced, basal cyclin D1 expression was elevated, and p27 regulation was unaffected.

DISCUSSION

Using a conditionally activated form of ROCK, in combination with the potent ROCK inhibitor Y-27632, we have determined that ROCK activation leads to increased levels of cyclin A and cyclin D1 and decreased levels of p27 in NIH 3T3 fibroblasts (Fig. 8B). Each of these pathways operated independently, since siRNA-mediated knockdown of each protein did not alter the expression of the others (Fig. 6). Also, MEK inhibition blocked ROCK-ER induction of cyclin D1 (Fig. 4D) and cell cycle progression (Fig. 2B) without affecting the cyclin A or p27 response (Fig. 4D), indicating that these proteins are not regulated by the MAPK pathway, cyclin D1 expression, or progression into S phase. The induction of cyclin A by ROCK-ER was reduced by siRNA-mediated knockdown of LIMK2, which also resulted in elevated basal cyclin D1 expression (Fig. 8A). However, given that cyclin A knockdown did not elevate basal cyclin D1 (Fig. 6A), these results suggest that the cyclin D1 regulation was independent of cyclin A and are consistent with the proposed role of LIMK in repressing the induction of cyclin D1 transcription by Rac and/or Cdc42 (58).

It has previously been shown that active Rho influences cell cycle progression via the regulation of a number of cell cycle regulatory proteins. One mechanism is through the regulation of p27. Inhibition of Rho function has been reported to elevate

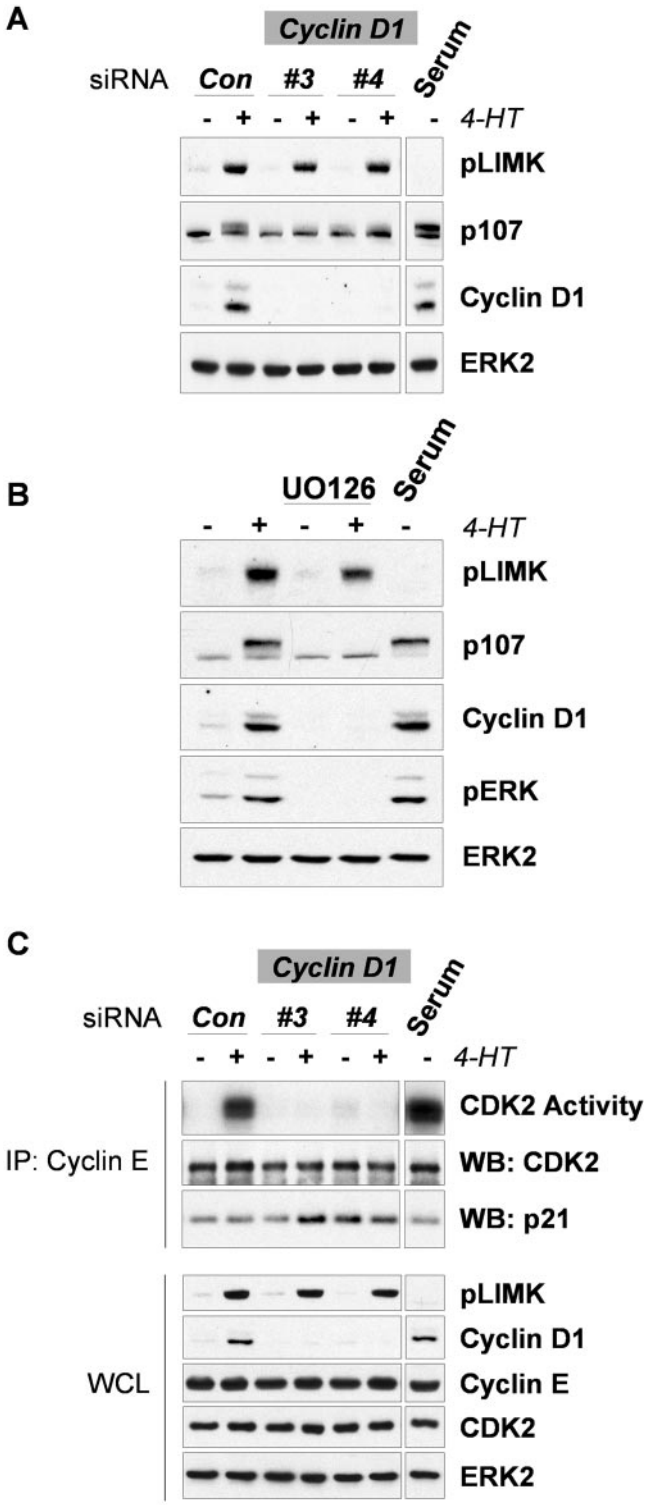

FIG. 7. Cyclin D1 knockdown inhibits ROCK-ER-induced p107 phosphorylation and cyclin E-associated CDK2 activity. (A) ROCK-ER-expressing NIH 3T3 cells were transfected with control nontargeting (*Con*) siRNA or siRNA duplexes targeting *Cyclin D1*. At 24 h posttransfection, cells were trypsinized and replated on plastic tissue culture dishes. Subconfluent cells were serum starved and then either left untreated or treated with 1 μM 4-HT for 16 h. Whole-cell lysates were immunoblotted for phospho-LIMK, p107, and cyclin D1. Equal protein loading was confirmed by blotting with an antibody against ERK2. Results of a representative experiment of two determinations with similar results are shown. (B) Serum-starved, ROCK-ER-expressing NIH 3T3 cells were either left untreated or treated with 1 μM 4-HT for 16 h, either in the presence or in the absence of 10 μM U0126. Cell lysates were immunoblotted for phospho-LIMK, p107, cyclin D1, and phospho-ERK. Equal protein loading was verified by blotting with an antibody against ERK2. Results of a representative experiment of two determinations with similar results are shown. (C) ROCK-ER-expressing NIH 3T3 cells were transfected with control nontargeting (*Con*) siRNA or siRNA duplexes targeting *Cyclin D1*. At 24 h posttransfection, cells were trypsinized and replated on plastic tissue culture dishes. Subconfluent cells were serum starved and then either left untreated or treated with 1 μM 4-HT for 16 h. Whole-cell lysates (WCL, lower half) were immunoblotted for phospho-LIMK, cyclin D1, cyclin E, and CDK2. Equal protein loading was confirmed by blotting with ERK2 antibody. Cyclin E was immunoprecipitated (IP), and associated CDK activity was assayed in vitro with histone H1 as the substrate. Cyclin E-associated CDK2 and p21 were revealed by Western blotting (WB) the immunoprecipitated complexes that had been assayed for kinase activity. Results of a representative experiment of two determinations with similar results are shown.

p27 protein levels (26, 27, 40, 56, 81), whereas expression of active Rho decreased p27 (27, 40, 56). Although inhibition of ROCK activity with Y-27632 has been associated with impaired p27 down-regulation (22, 29, 30, 35, 64), it has not been clear whether the effect of ROCK inhibition on p27 levels is direct or a secondary consequence of decreased proliferation. In this study, we show that the selective activation of ROCK is sufficient to lower p27 levels, independently of MAPK activation or the induction of cyclin A or cyclin D1. In addition to the possibility that ROCK activity promotes p27 protein degradation, ROCK might repress p27 translation since Rho inhibition was shown to increase p27 mRNA translation through a Rho-responsive element in the 3′-untranslated region (78). More research is required to elucidate the mechanism of ROCK-induced p27 down-regulation.

Another mechanism that contributes to cell cycle regulation is Rho-mediated suppression of p21 transcription (1, 4, 23, 42, 50). However, ROCK function was found not to be required for p21 suppression by Rho in normal and Ras-transformed fibroblasts or in colon carcinoma cell lines (57, 59, 61). Our results indicate that ROCK activation increased p21 levels (Fig. 3A), likely as a result of Ras/MAPK activation (43) and possibly from increased cyclin D1 leading to p21 protein stabilization (13). However, the induction of p21 was observed under conditions that were permissive for G_1/S progression (Fig. 2), indicating that the extent of p21 induction was compatible with proliferation.

In addition to the regulation of CDKIs, Rho has been reported to influence cyclin levels. Rho and ROCK are necessary for Ras-GTP loading and sustained ERK activation, leading to increased cyclin D1 transcription following growth factor stimulation, through their contribution to the maintenance of actin stress fibers (57, 71, 83). Our data indicate that the sustained activation of ROCK, which leads to the formation of strong actin stress fibers (Fig. 1D), is sufficient to induce cell cycle progression (Fig. 2), Ras-GTP loading (Fig. 4B), ERK activation (Fig. 4D), and increased MAPK-dependent cyclin D1 expression (Fig. 4D). A model has been proposed that suggests that ROCK activation of LIMK results in the transcriptional

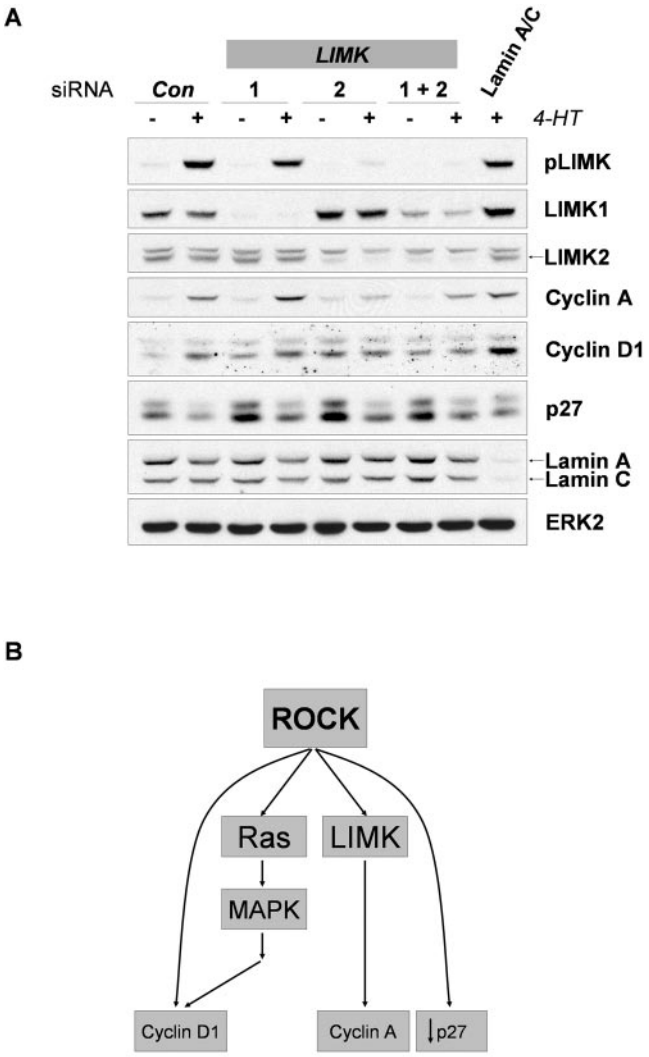

FIG. 8. LIMK2 knockdown abrogates ROCK-ER-induced cyclin A upregulation. (A) ROCK-ER-expressing NIH 3T3 cells were transfected with control nontargeting (*Con*) siRNA or siRNA(s) against *LIMK1*, *LIMK2*, *LIMK1* and *-2* in combination, or *Lamin A/C*. At 24 h posttransfection, cells were trypsinized and replated on plastic tissue culture dishes. Subconfluent cells were serum starved and then either left untreated or treated with 1 μM 4-HT for 16 h. Whole-cell lysates were immunoblotted for phospho-LIMK, LIMK 1, LIMK 2, cyclin A, cyclin D1, p27, and lamin A/C. Blotting for ERK2 confirmed equal protein loading. Results of a representative experiment of three determinations with similar results are shown. (B) Model of ROCK regulation of cell cycle proteins. ROCK-ER stimulation leads to activation of Ras and MAPK, which, along with an additional independent pathway, leads to elevation of cyclin D1 expression. Cyclin A is independently elevated in response to ROCK activation via LIMK2. Reduced p27 protein levels were observed following ROCK activation, independent of the effect on cyclin D1, cyclin A, or cell cycle progression.

repression of Rac/Cdc42-induced cyclin D1 transcription and that inhibition of ROCK relieves the LIMK-mediated repression (58). We did observe that siRNA-mediated knockdown of LIMK2 resulted in increased basal cyclin D1 expression (Fig. 8A), suggesting that LIMK-mediated transcriptional repression may be independent of its ROCK-regulated catalytic activity, possibly acting through direct actions on nuclear targets (58).

NIH 3T3 cells transformed with a constitutively active version of RhoA were reported to have elevated levels of cyclin A by microarray analysis (73). In addition, inhibition of Rho or ROCK function in atrial myofibroblasts inhibited proliferation and blocked cyclin A expression (52). However, the possibility exists that cyclin A expression was influenced indirectly through effects on cell cycle regulation and not necessarily a direct or specific RhoA- or ROCK-induced phenomenon in these studies. We found that ROCK-ER activation led to increased cyclin A levels, which were independent of MAPK activity, p107 phosphorylation, or cyclin D1 and p27 changes. We found that disruption of the actin cytoskeleton resulted in elevated basal cyclin A expression, which could be further increased by ROCK activation in CCD- and LTB-treated cells (Fig. 4A). We believe that in SWA- and Jasp-treated cells, the elevated basal cyclin A expression was maximal such that ROCK activation could not elevate expression further. These data suggest that the regulation of cyclin A by ROCK is independent of actin regulation, in direct contrast to the regulation of cyclin D1, which appears to require functional actin cytoskeletal structures. Knockdown of LIMK2 resulted in decreased cyclin A induction by ROCK-ER (Fig. 8A). Combining these results suggests that cyclin A induction by ROCK is mediated by an actin-independent LIMK-mediated pathway, similar to the LIMK-mediated, actin-independent repression of Rac/Cdc42-stimulated cyclin D1 transcription (58). It remains to be determined whether nuclear translocation of LIMK2 is required for cyclin A regulation, as has been shown for LIMK-mediated repression of cyclin D1 transcription (58).

Treatment of endothelial cells with tumor necrosis factor alpha was recently reported to result in cyclin A transcriptional repression, mediated by ROCK phosphorylation of ezrin and consequent nuclear translocation of ezrin, where it repressed transcription by binding to cell cycle homology region repressor elements within the cyclin A promoter (37). In contrast, we found that ROCK activation actually increased cyclin A expression (Fig. 4A) and have previously reported no change in ezrin phosphorylation under conditions where tumor necrosis factor alpha treatment of NIH 3T3 cells led to increased ROCK-mediated phosphorylation of LIMK and MLC2 (16). The differences between these findings may be the consequence of cell-specific factors.

It has been reported that Rho activity is necessary for cyclin E expression in rat astrocytes (72). Our findings indicate that ROCK activity is neither necessary nor sufficient for cyclin E expression (Fig. 3A), suggesting that there may also be cell type-specific factors that contribute to cyclin E regulation.

We show in this study that ROCK activation is sufficient to induce G_1/S-phase cell cycle progression in $p16^{-/-}$ NIH 3T3 mouse fibroblasts, which is associated with increased levels of cyclin D1 and cyclin A and lowered p27 levels. Previous research has implicated Rho and ROCK signaling in the regulation of these proteins; however, the experimental design has not always allowed effects on cell cycle regulatory proteins to be dissociated from global effects that result from cell cycle arrest. Also, given that many of these previous studies have relied upon inhibition of ROCK, it has not always been possible to conclude that ROCK is both necessary and sufficient for the observed

effects on cell cycle regulators. Through the selective activation of conditionally regulated ROCK-ER, we found that (i) ROCK stimulation of actin stress fibers is sufficient to activate the Ras/MAPK pathway and elevate cyclin D1 expression; (ii) ROCK regulation of cyclin D1, that of cyclin A, and that of p27 are independent events; and (iii) cyclin A is regulated by ROCK through a previously uncharacterized LIMK-mediated mechanism. These results broaden our knowledge of Rho and ROCK regulation of the cell cycle, which may help in the design of anticancer therapies that target this signaling pathway (79). Two questions that must be examined in more detail to determine whether these results are broadly applicable are (i) whether human cells behave in the same manner as mouse cells and (ii) whether the absence of p16 in NIH 3T3 cells contributes to the ability of ROCK to promote cell cycle progression.

ACKNOWLEDGMENTS

We thank Sharon Benzeno, Alan Diehl, and Andrew Gladden for useful discussions and reagents. We also thank Chris Marshall (ICR, London, United Kingdom) for anti-ERK2 antibody and Martin McMahon (San Francisco, CA) for the Raf-ER plasmid.

This research was supported by Cancer Research UK and a grant to M.F.O. from the NIH (CA030721).

REFERENCES

1. **Adnane, J., F. A. Bizouarn, Y. Qian, A. D. Hamilton, and S. M. Sebti.** 1998. $p21^{WAF1/CIP1}$ is upregulated by the geranylgeranyltransferase I inhibitor GGTI-298 through a transforming growth factor beta- and Sp1-responsive element: involvement of the small GTPase RhoA. Mol. Cell. Biol. **18:**6962–6970.
2. **Alessi, D. R., A. Cuenda, P. Cohen, D. T. Dudley, and A. R. Saltiel.** 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. **270:**27489–27494.
3. **Assoian, R. K., and X. Zhu.** 1997. Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression. Curr. Opin. Cell Biol. **9:**93–98.
4. **Auer, K. L., J. S. Park, P. Seth, R. J. Coffey, G. Darlington, A. Abo, M. McMahon, R. A. Depinho, P. B. Fisher, and P. Dent.** 1998. Prolonged activation of the mitogen-activated protein kinase pathway promotes DNA synthesis in primary hepatocytes from p21Cip-1/WAF1-null mice, but not in hepatocytes from p16INK4a-null mice. Biochem. J. **336:**551–560.
5. **Bastow, E. R., K. J. Lamb, J. C. Lewthwaite, A. C. Osborne, E. Kavanagh, C. P. D. Wheeler-Jones, and A. A. Pitsillides.** 2005. Selective activation of the MEK-ERK pathway is regulated by mechanical stimuli in forming joints and promotes pericellular matrix formation. J. Biol. Chem. **280:**11749–11758.
6. **Blanchard, J. M.** 2000. Cyclin A2 transcriptional regulation: modulation of cell cycle control at the G_1/S transition by peripheral cues. Biochem. Pharmacol. **60:**1179–1184.
7. **Bottazzi, M. E., M. Buzzai, X. Zhu, C. Desdouets, C. Brechot, and R. K. Assoian.** 2001. Distinct effects of mitogens and the actin cytoskeleton on CREB and pocket protein phosphorylation control the extent and timing of cyclin A promoter activity. Mol. Cell. Biol. **21:**7607–7616.
8. **Bubb, M. R., I. Spector, A. D. Bershadsky, and E. D. Korn.** 1995. Swinholide A is a microfilament disrupting marine toxin that stabilizes actin dimers and severs actin filaments. J. Biol. Chem. **270:**3463–3466.
9. **Bubb, M. R., I. Spector, B. B. Beyer, and K. M. Fosen.** 2000. Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain in vivo observations. J. Biol. Chem. **275:**5163–5170.
10. **Cechin, S. R., P. R. Dunkley, and R. Rodnight.** 2005. Signal transduction mechanisms involved in the proliferation of C6 glioma cells induced by lysophosphatidic acid. Neurochem. Res. **30:**603–611.
11. **Cheng, T. L., M. Symons, and T. S. Jou.** 2004. Regulation of anoikis by Cdc42 and Rac1. Exp. Cell Res. **295:**497–511.
12. **Coleman, M. L., C. J. Marshall, and M. F. Olson.** 2004. RAS and RHO GTPases in G_1-phase cell-cycle regulation. Nat. Rev. Mol. Cell. Biol. **5:**355–366.
13. **Coleman, M. L., C. J. Marshall, and M. F. Olson.** 2003. Ras promotes $p21^{Waf1/Cip1}$ protein stability via a cyclin D1-imposed block in proteasome-mediated degradation. EMBO J. **22:**2036–2046.
14. **Collins, N. L., M. J. Reginato, J. K. Paulus, D. C. Sgroi, J. Labaer, and J. S. Brugge.** 2005. G_1/S cell cycle arrest provides anoikis resistance through Erk-mediated Bim suppression. Mol. Cell. Biol. **25:**5282–5291.
15. **Coniglio, S. J., T. S. Jou, and M. Symons.** 2001. Rac1 protects epithelial cells against anoikis. J. Biol. Chem. **276:**28113–28120.
16. **Croft, D. R., M. L. Coleman, S. Li, D. Robertson, T. Sullivan, C. L. Stewart, and M. F. Olson.** 2005. Actin-myosin-based contraction is responsible for apoptotic nuclear disintegration. J. Cell Biol. **168:**245–255.
17. **Croft, D. R., and M. F. Olson.** 2006. Conditional regulation of a ROCK-estrogen receptor fusion protein. Methods Enzymol. **406:**541–553.
18. **Croft, D. R., E. Sahai, G. Mavria, S. Li, J. Tsai, W. M. Lee, C. J. Marshall, and M. F. Olson.** 2004. Conditional ROCK activation in vivo induces tumor cell dissemination and angiogenesis. Cancer Res. **64:**8994–9001.
19. **Delaney, A. M., J. A. Printen, H. Chen, E. B. Fauman, and D. T. Dudley.** 2002. Identification of a novel mitogen-activated protein kinase kinase activation domain recognized by the inhibitor PD 184352. Mol. Cell. Biol. **22:**7593–7602.
20. **Gladden, A. B., and J. A. Diehl.** 2005. Location, location, location: the role of cyclin D1 nuclear localization in cancer. J. Cell. Biochem. **96:**906–913.
21. **Gomez del Pulgar, T., S. A. Benitah, P. F. Valeron, C. Espina, and J. C. Lacal.** 2005. Rho GTPase expression in tumourigenesis: evidence for a significant link. Bioessays **27:**602–613.
22. **Guerin, P., V. Sauzeau, M. Rolli-Derkinderen, O. Al Habbash, E. Scalbert, D. Crochet, P. Pacaud, and G. Loirand.** 2005. Stent implantation activates RhoA in human arteries: inhibitory effect of rapamycin. J. Vasc. Res. **42:**21–28.
23. **Han, S., N. Sidell, and J. Roman.** 2005. Fibronectin stimulates human lung carcinoma cell proliferation by suppressing p21 gene expression via signals involving Erk and Rho kinase. Cancer Lett. **219:**71–81.
24. **Harvey, S. A., S. C. Anderson, and N. SundarRaj.** 2004. Downstream effects of ROCK signaling in cultured human corneal stromal cells: microarray analysis of gene expression. Investig. Ophthalmol. Vis. Sci. **45:**2168–2176.
25. **He, H., J. Pannequin, J. P. Tantiongco, A. Shulkes, and G. S. Baldwin.** 2005. Glycine-extended gastrin stimulates cell proliferation and migration through a Rho- and ROCK-dependent pathway, not a Rac/Cdc42-dependent pathway. Am. J. Physiol. Gastrointest. Liver Physiol. **289:**G478–G488.
26. **Hirai, A., S. Nakamura, Y. Noguchi, T. Yasuda, M. Kitagawa, I. Tatsuno, T. Oeda, K. Tahara, T. Terano, S. Narumiya, L. D. Kohn, and Y. Saito.** 1997. Geranylgeranylated rho small GTPase(s) are essential for the degradation of p27Kip1 and facilitate the progression from G_1 to S phase in growth-stimulated rat FRTL-5 cells. J. Biol. Chem. **272:**13–16.
27. **Hu, W., C. J. Bellone, and J. J. Baldassare.** 1999. RhoA stimulates $p27^{Kip}$ degradation through its regulation of cyclin E/CDK2 activity. J. Biol. Chem. **274:**3396–3401.
28. **Ishida, K., H. Nagahara, T. Kogiso, T. Aso, N. Hayashi, and T. Akaike.** 2003. Cell adhesion aside from integrin system can abrogate anoikis in rat liver cells by down-regulation of FasL expression, not by activation of PI-3K/Akt and ERK signaling pathway. Biochem. Biophys. Res. Commun. **300:**201–208.
29. **Iso, Y., H. Suzuki, T. Sato, M. Shoji, N. Shimizu, M. Shibata, S. Koba, E. Geshi, and T. Katagiri.** 2006. Rho-kinase inhibitor suppressed restenosis in porcine coronary balloon angioplasty. Int. J. Cardiol. **106:**103–110.
30. **Iwamoto, H., M. Nakamuta, S. Tada, R. Sugimoto, M. Enjoji, and H. Nawata.** 2000. A p160ROCK-specific inhibitor, Y-27632, attenuates rat hepatic stellate cell growth. J. Hepatol. **32:**762–770.
31. **Kalisch, B. E., C. S. Demeris, M. Ishak, and R. J. Rylett.** 2003. Modulation of nerve growth factor-induced activation of MAP kinase in PC12 cells by inhibitors of nitric oxide synthase. J. Neurochem. **87:**1321–1332.
32. **Kamai, T., K. Arai, S. Sumi, T. Tsujii, M. Honda, T. Yamanishi, and K. I. Yoshida.** 2002. The rho/rho-kinase pathway is involved in the progression of testicular germ cell tumour. BJU Int. **89:**449–453.
33. **Kamai, T., T. Tsujii, K. Arai, K. Takagi, H. Asami, Y. Ito, and H. Oshima.** 2003. Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin. Cancer Res. **9:**2632–2641.
34. **Kamiyama, M., K. Utsunomiya, K. Taniguchi, T. Yokota, H. Kurata, N. Tajima, and K. Kondo.** 2003. Contribution of Rho A and Rho kinase to platelet-derived growth factor-BB-induced proliferation of vascular smooth muscle cells. J. Atheroscler. Thromb. **10:**117–123.
35. **Kanda, T., K. Hayashi, S. Wakino, K. Homma, K. Yoshioka, K. Hasegawa, N. Sugano, S. Tatematsu, I. Takamatsu, T. Mitsuhashi, and T. Saruta.** 2005. Role of Rho-kinase and p27 in angiotensin II-induced vascular injury. Hypertension **45:**724–729.
36. **Kaneko, K., K. Satoh, A. Masamune, A. Satoh, and T. Shimosegawa.** 2002. Expression of ROCK-1 in human pancreatic cancer: its down-regulation by morpholino oligo antisense can reduce the migration of pancreatic cancer cells in vitro. Pancreas **24:**251–257.
37. **Kishore, R., G. Qin, C. Luedemann, E. Bord, A. Hanley, M. Silver, M. Gavin, D. Goukassian, and D. W. Losordo.** 2005. The cytoskeletal protein ezrin regulates EC proliferation and angiogenesis via TNF-α-induced transcriptional repression of cyclin A. J. Clin. Investig. **115:**1785–1796.
38. **Kleer, C. G., K. L. van Golen, Y. Zhang, Z. F. Wu, M. A. Rubin, and S. D. Merajver.** 2002. Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability. Am. J. Pathol. **160:**579–584.
39. **Landis, M. W., B. S. Pawlyk, T. Li, P. Sicinski, and P. W. Hinds.** 2006. Cyclin

D1-dependent kinase activity in murine development and mammary tumorigenesis. Cancer Cell **9:**13–22.
40. **Laufs, U., D. Marra, K. Node, and J. K. Liao.** 1999. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle proliferation by preventing rho GTPase-induced down-regulation of $p27^{Kip1}$. J. Biol. Chem. **274:**21926–21931.
41. **Leung, T., X. Q. Chen, E. Manser, and L. Lim.** 1996. The p160 RhoA-binding kinase ROKα is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol. Cell. Biol. **16:**5313–5327.
42. **Liberto, M., D. Cobrinik, and A. Minden.** 2002. Rho regulates $p21^{CIP1}$, cyclin D1, and checkpoint control in mammary epithelial cells. Oncogene **21:**1590–1599.
43. **Liu, Y., J. L. Martindale, M. Gorospe, and N. J. Holbrook.** 1996. Regulation of $p21^{WAF1/CIP1}$ expression through mitogen-activated protein kinase signaling pathway. Cancer Res. **56:**31–35.
44. **Liu, Y., Y. J. Suzuki, R. M. Day, and B. L. Fanburg.** 2004. Rho kinase-induced nuclear translocation of ERK1/ERK2 in smooth muscle cell mitogenesis caused by serotonin. Circ. Res. **95:**579–586.
45. **Mallat, Z., A. Gojova, V. Sauzeau, V. Brun, J. S. Silvestre, B. Esposito, R. Merval, H. Groux, G. Loirand, and A. Tedgui.** 2003. Rho-associated protein kinase contributes to early atherosclerotic lesion formation in mice. Circ. Res. **93:**884–888.
46. **McMullan, R., S. Lax, V. H. Robertson, D. J. Radford, S. Broad, F. M. Watt, A. Rowles, D. R. Croft, M. F. Olson, and N. A. Hotchin.** 2003. Keratinocyte differentiation is regulated by the Rho and ROCK signaling pathway. Curr. Biol. **13:**2185–2189.
47. **Montagnoli, A., F. Fiore, E. Eytan, A. C. Carrano, G. F. Draetta, A. Hershko, and M. Pagano.** 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. **13:**1181–1189.
48. **Nishimaki, H., K. Kasai, K. Kozaki, T. Takeo, H. Ikeda, S. Saga, M. Nitta, and G. Itoh.** 2004. A role of activated Sonic hedgehog signaling for the cellular proliferation of oral squamous cell carcinoma cell line. Biochem. Biophys. Res. Commun. **314:**313–320.
49. **Olson, M. F., A. Ashworth, and A. Hall.** 1995. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G_1. Science **269:**1270–1272.
50. **Olson, M. F., H. F. Paterson, and C. J. Marshall.** 1998. Signals from Ras and Rho GTPases interact to regulate expression of $p21^{Waf1/Cip1}$. Nature **394:**295–299.
51. **Perona, R., P. Esteve, B. Jimenez, R. P. Ballestero, S. Ramon y Cajal, and J. C. Lacal.** 1993. Tumorigenic activity of rho genes from Aplysia californica. Oncogene **8:**1285–1292.
52. **Porter, K. E., N. A. Turner, D. J. O'Regan, A. J. Balmforth, and S. G. Ball.** 2004. Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. Cardiovasc. Res. **61:**745–755.
53. **Rees, R. W., N. A. Foxwell, D. J. Ralph, P. D. Kell, S. Moncada, and S. Cellek.** 2003. Y-27632, a Rho-kinase inhibitor, inhibits proliferation and adrenergic contraction of prostatic smooth muscle cells. J. Urol. **170:**2517–2522.
54. **Ridley, A. J., and A. Hall.** 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70:**389–399.
55. **Riento, K., and A. J. Ridley.** 2003. Rocks: multifunctional kinases in cell behaviour. Nat. Rev. Mol. Cell. Biol. **4:**446–456.
56. **Rivard, N., M. J. Boucher, C. Asselin, and G. L'Allemain.** 1999. MAP kinase cascade is required for p27 downregulation and S phase entry in fibroblasts and epithelial cells. Am. J. Physiol. **277:**C652–C664.
57. **Roovers, K., and R. K. Assoian.** 2003. Effects of rho kinase and actin stress fibers on sustained extracellular signal-regulated kinase activity and activation of G_1 phase cyclin-dependent kinases. Mol. Cell. Biol. **23:**4283–4294.
58. **Roovers, K., E. A. Klein, P. Castagnino, and R. K. Assoian.** 2003. Nuclear translocation of LIM kinase mediates Rho-Rho kinase regulation of cyclin D1 expression. Dev. Cell **5:**273–284.
59. **Sahai, E., T. Ishizaki, S. Narumiya, and R. Treisman.** 1999. Transformation mediated by RhoA requires activity of ROCK kinases. Curr. Biol. **9:**136–145.
60. **Sahai, E., and C. J. Marshall.** 2002. RHO-GTPases and cancer. Nat. Rev. Cancer **2:**133–142.
61. **Sahai, E., M. F. Olson, and C. J. Marshall.** 2001. Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increased motility. EMBO J. **20:**755–766.
62. **Sampath, P., and T. D. Pollard.** 1991. Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. Biochemistry **30:**1973–1980.
63. **Sauzeau, V., E. Le Mellionnec, J. Bertoglio, E. Scalbert, P. Pacaud, and G. Loirand.** 2001. Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. Circ. Res. **88:**1102–1104.
64. **Sawada, N., H. Itoh, K. Ueyama, J. Yamashita, K. Doi, T. H. Chun, M. Inoue, K. Masatsugu, T. Saito, Y. Fukunaga, S. Sakaguchi, H. Arai, N. Ohno, M. Komeda, and K. Nakao.** 2000. Inhibition of rho-associated kinase results in suppression of neointimal formation of balloon-injured arteries. Circulation **101:**2030–2033.
65. **Seasholtz, T. M., M. Majumdar, D. D. Kaplan, and J. H. Brown.** 1999. Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. Circ. Res. **84:**1186–1193.
66. **Seibold, S., D. Schurle, A. Heinloth, G. Wolf, M. Wagner, and J. Galle.** 2004. Oxidized LDL induces proliferation and hypertrophy in human umbilical vein endothelial cells via regulation of $p27^{Kip1}$ expression: role of RhoA. J. Am. Soc. Nephrol. **15:**3026–3034.
67. **Sherr, C. J.** 2000. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. **60:**3689–3695.
68. **Sorensen, S. D., O. Nicole, R. D. Peavy, L. M. Montoya, C. J. Lee, T. J. Murphy, S. F. Traynelis, and J. R. Hepler.** 2003. Common signaling pathways link activation of murine PAR-1, LPA, and S1P receptors to proliferation of astrocytes. Mol. Pharmacol. **64:**1199–1209.
69. **Spector, I., N. R. Shochet, Y. Kashman, and A. Groweiss.** 1983. Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. Science **219:**493–495.
70. **Spence, H. J., L. McGarry, C. S. Chew, N. O. Carragher, L. A. Scott-Carragher, Z. Yuan, D. R. Croft, M. F. Olson, M. Frame, and B. W. Ozanne.** 2006. AP-1 differentially expressed proteins Krp1 and fibronectin cooperatively enhance Rho-ROCK-independent mesenchymal invasion by altering the function, localization, and activity of nondifferentially expressed proteins. Mol. Cell. Biol. **26:**1480–1495.
71. **Swant, J. D., B. E. Rendon, M. Symons, and R. A. Mitchell.** 2005. Rho GTPase-dependent signaling is required for macrophage migration inhibitory factor-mediated expression of cyclin D1. J. Biol. Chem. **280:**23066–23072.
72. **Tanaka, T., I. Tatsuno, Y. Noguchi, D. Uchida, T. Oeda, S. Narumiya, T. Yasuda, H. Higashi, M. Kitagawa, K. Nakayama, Y. Saito, and A. Hirai.** 1998. Activation of cyclin-dependent kinase 2 (Cdk2) in growth-stimulated rat astrocytes. Geranylgeranylated Rho small GTPase(s) are essential for the induction of cyclin E gene expression. J. Biol. Chem. **273:**26772–26778.
73. **Teramoto, H., R. L. Malek, B. Behbahani, M. D. Castellone, N. H. Lee, and J. S. Gutkind.** 2003. Identification of H-Ras, RhoA, Rac1 and Cdc42 responsive genes. Oncogene **22:**2689–2697.
74. **Tharaux, P. L., R. C. Bukoski, P. N. Rocha, S. D. Crowley, P. Ruiz, C. Nataraj, D. N. Howell, K. Kaibuchi, R. F. Spurney, and T. M. Coffman.** 2003. Rho kinase promotes alloimmune responses by regulating the proliferation and structure of T cells. J. Immunol. **171:**96–105.
75. **Uehata, M., T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, and S. Narumiya.** 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature **389:**990–994.
76. **van Rossum, G. S. A. T., R. Klooster, H. van den Bosch, A. J. Verkleij, and J. Boonstra.** 2001. Phosphorylation of p42/44MAPK by various signal transduction pathways activates cytosolic phospholipase A2 to variable degrees. J. Biol. Chem. **276:**28976–28983.
77. **Vichalkovski, A., K. Baltensperger, D. Thomann, and H. Porzig.** 2005. Two different pathways link G-protein-coupled receptors with tyrosine kinases for the modulation of growth and survival in human hematopoietic progenitor cells. Cell Signal. **17:**447–459.
78. **Vidal, A., S. S. Millard, J. P. Miller, and A. Koff.** 2002. Rho activity can alter the translation of p27 mRNA and is important for RasV12-induced transformation in a manner dependent on p27 status. J. Biol. Chem. **277:**16433–16440.
79. **Walker, K., and M. F. Olson.** 2005. Targeting Ras and Rho GTPases as opportunities for cancer therapeutics. Curr. Opin. Genet. Dev. **15:**62–68.
80. **Wang, G., A. Woods, S. Sabari, L. Pagnotta, L. A. Stanton, and F. Beier.** 2004. RhoA/ROCK signaling suppresses hypertrophic chondrocyte differentiation. J. Biol. Chem. **279:**13205–13214.
81. **Weber, J. D., W. Hu, S. C. Jefcoat, Jr., D. M. Raben, and J. J. Baldassare.** 1997. Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G_1 progression through the independent regulation of cyclin D1 and p27. J. Biol. Chem. **272:**32966–32971.
82. **Weiss, R. H., E. A. Maga, and A. Ramirez.** 1998. MEK inhibition augments Raf activity, but has variable effects on mitogenesis, in vascular smooth muscle cells. Am. J. Physiol. Cell Physiol. **274:**C1521–C1529.
83. **Welsh, C. F., K. Roovers, J. Villanueva, Y. Liu, M. A. Schwartz, and R. K. Assoian.** 2001. Timing of cyclin D1 expression within G_1 phase is controlled by Rho. Nat. Cell Biol. **3:**950–957.
84. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon.** 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf→MEK→ERK pathway. Exp. Cell Res. **287:**325–338.
85. **Yamamoto, M., N. Marui, T. Sakai, N. Morii, S. Kozaki, K. Ikai, S. Imamura, and S. Narumiya.** 1993. ADP-ribosylation of the rhoA gene product by botulinum C3 exoenzyme causes Swiss 3T3 cells to accumulate in the G_1 phase of the cell cycle. Oncogene **8:**1449–1455.
86. **Zhao, Z., and S. A. Rivkees.** 2003. Rho-associated kinases play an essential

role in cardiac morphogenesis and cardiomyocyte proliferation. Dev. Dyn. **226:**24–32.

87. **Zhou, J., L. Q. Zhao, M. M. Xiong, X. Q. Wang, G. R. Yang, Z. L. Qiu, M. Wu, and Z. H. Liu.** 2003. Gene expression profiles at different stages of human esophageal squamous cell carcinoma. World J. Gastroenterol. **9:**9–15.
88. **Zhu, X.-H., H. Nguyen, H. D. Halicka, F. Traganos, and A. Koff.** 2004. Noncatalytic requirement for cyclin A-cdk2 in p27 turnover. Mol. Cell. Biol. **24:**6058–6066.
89. **Zugasti, O., W. Rul, P. Roux, C. Peyssonnaux, A. Eychene, T. F. Franke, P. Fort, and U. Hibner.** 2001. Raf-MEK-Erk cascade in anoikis is controlled by Rac1 and Cdc42 via Akt. Mol. Cell. Biol. **21:**6706–6717.